MECHANISM OF ENERGIZATION OF TRANSHYDROGENASE IN *Escherichia coli* MEMBRANES

by

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ABSTRACT

Low concentrations of the group IIA metals Mg^{2+}, Ca^{2+}, and Ba^{2+} stimulated energy-independent transhydrogenase activity. High concentrations of Mg^{2+} inhibited this activity. Transhydrogenase requires Mg^{2+}-complexed NADP(H) rather than free NADP(H) as its substrate. High concentrations of Mg^{2+}, however, may change the conformation of the enzyme to inhibit its enzymatic reaction by binding directly to the NADP(H) site.

Upon transhydrogenation between NADPH and 3-acetylpyridine dinucleotide, *E. coli* pyridine nucleotide transhydrogenase can establish a proton gradient across the cell membrane. The primary component of the proton gradient for energization of transhydrogenase was found to be the pH gradient and not the membrane potential. A similar conclusion was drawn for the ATP-driven transhydrogenase reactions.

In strains of *E. coli* that harbored plasmids to give the cells elevated levels of transhydrogenase, it was found that uncouplers stimulated the aerobic-driven transhydrogenase reaction. This is a chemiosmotic anomaly and is in contrast to the non-plasmid containing parent strains where uncouplers inhibited the activity. Further investigation revealed that the plasmid strains contained a much lower NADH oxidase activity than the non-plasmid strains and that neither KCN nor QNO can inhibit the aerobic-dependent activity in both types of strains even though they were effective in blocking the respiratory chain. These effects prompted us to inquire whether the anomaly was due to differences in the respiratory chain, but no differences were found between the NADH dehydrogenase activities, quinone and cytochrome contents of the plasmid and non-plasmid strains.

The bacterial cells with amplified transhydrogenases induce extra intracellular tubular membrane structures to accomodate the extra proteins (Clark, D.M., *Pyridine Nucleotide Transhydrogenase*, PhD thesis, University of British Columbia, 1986). Separation of the *E. coli* membrane vesicles on a shallow sucrose gradient,

however, did not reveal any differences between the vesicles of the plasmid and non-plasmid strains. Therefore, it seems unlikely that the anomaly is due to the plasmid strains performing a unique form of energization on these induced structures.

Finally, it was established by SDS-PAGE and Western blot using anti-transhydrogenase antisera that the plasmid strains express a much higher level of transhydrogenase enzymes in their cell membranes than do the non-plasmid strains.

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AcNAD(H)	3-Acetylpyridine adenine dinucleotide
ADP	Adenosine-5'-diphosphate
Asc	Ascorbate
ATP	Adenosine-5'-triphosphate
CCCP	Carbonyl cyanide *m*-chlorophenylhydrazone
DCCD	N, N'-dicyclohexylcarbodiimide
dh	Dehydrogenase
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
d-NADH	Deamino nicotinamide adenine dinucleotide
EDTA	(Ethylenedinitrilo)-tetraacetic acid
E. coli	Escherichia coli
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
F_1	Enzymatic unit of ATPase
F_o	Proton channel unit of ATPase
Hepes	N-2-hydroxyethylpiperazine
NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate
Nig.	Nigericin
PAB	Penassay broth
PAGE	Polyacrylamide gel electrophoresis
P_i	inorganic phosphate
PMS	Phenazine Methosulfate
Q	Quinone

QNO	2-n-Heptyl-4-hydroxyquinoline N-oxide
Q_1	Ubiquinone 1
SDS	Sodium dodecyl sulfate
TBTC	Tri-n-butyltin chloride
TCS	3, 3', 4', 5-Tetrachlorosalicylanilide
TEMED	N,N,N',N'-tetraacetic acid
TMAO	Trimethylamine-N-oxide
TPTC	Triphenyltin chloride
Tris	Tris (hydroxymethyl)-aminoethane
Val.	Valinomycin
v/v	Volume to volume ratio
w/v	Weight to volume ratio
w/w	Weight to weight ratio
x g	Times gravity
ΔE	Extinction coefficient
Δp	Proton motive force
ΔpH	Difference in pH across the membrane
$\Delta \mu_H$	Proton electrochemical potential
$\Delta \psi$	Electrical potential

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> For with much wisdom, comes much sorrow;
> the more knowledge, the more grief.
>
> Ecclesiastes 1:18

I INTRODUCTION

Over the past 40 years, much work has been done to elucidate how energy made available by the oxidation of substrates or by the absorption of light is used to synthesize ATP or to actively transport ions across the membrane. The "energy transducing" membranes which perform these functions vary from the plasma membrane of bacteria or blue green algae, to the thylakoid membrane of chloroplasts and the inner membrane of mitochondria (55). Yet in spite of this large diversity, sufficient similarities exist between these systems to allow uniform study of oxidative phosphorylation.

In a simplified form, most energy transducing membranes can be viewed as consisting of two main protein assemblies. The first is the ATP synthase. Its function is to catalyze the energy requiring synthesis of ATP from ADP and P_i or visa versa. It is ubiquitous to most energy transducing membranes, though some minor alterations in structure may exist between organisms (89). The nature of the second assembly, the electron transport chain, is more complex and varies a great deal from system to system. One example is the mitochondrion which has a respiratory chain that can be operationally defined to four distinct complexes plus ubiquinone and cytochrome c. It oxidizes NADH as its primary energy source and reduces oxygen as its terminal electron acceptor. Most mitochondria have this pathway only (90). In contrast, *E coli* can synthesize several different respiratory chains depending on the carbon source, terminal electron acceptor, and growth conditions. Often, more than one respiratory chain is synthesized at a time (29). The chloroplast and photosynthetic bacteria also have complex and varied respiratory chains but they use light as their primary energy source (55). In all, the final product of the second assembly is to provide energy which can be used to drive "uphill" reactions such as ATP synthesis for the first assembly.

The mystery which remains to be solved is the nature of the "energy-transducing intermediate" linking these pairs of protein assemblies. Many hypotheses have been proposed but none have yet provided a satisfactory explanation. Nevertheless, from time to time during the development of ideas about oxidative phosphorylation, one or two concepts have dominated the thinking of the workers in the field. For example, hypotheses involving chemical coupling, conformational coupling, chemiosmosis, and localized protons have been proposed and they will be described in greater detail below.

A. Theories of Oxidative Phosphorylation

1. Early ideas of energy coupling - Chemical and Conformational Hypothesis

In 1939, Warburg and Christian (1) put forward the idea that ATP is synthesized by high energy phosphorylated intermediates during glycolysis. In 1941, Lipman established that ATP is the unit of "energy currency" in the cell. With the success of these two concepts, the general opinion was that a system of high energy compounds was the only convertible and transportable form of energy in the living cell. Therefore, when researchers were faced with the problem of oxidative phosphorylation, they naturally sought a high energy intermediate.

The first results indicated that a high energy non-phosphorylated intermediate may be involved in oxidative phosphorylation. In experiments with mitochondria, it was demonstrated that substrates can be oxidized in the complete absence of ADP and phosphate. These observations, in direct contrast with glycolysis, prompted Slater (3) in 1953 to suggest an unknown high energy compound (x~y), possibly a thioester, to be the ATP precursor in oxidative phosphorylation. This hypothesis seemed for a time to receive support from experimental evidence. Lardy et al. (4) hypothesized that oligomycin prevented energy transfer between x~y and ATP. Furthermore, x~y produced by the second and third energy coupling sites of the

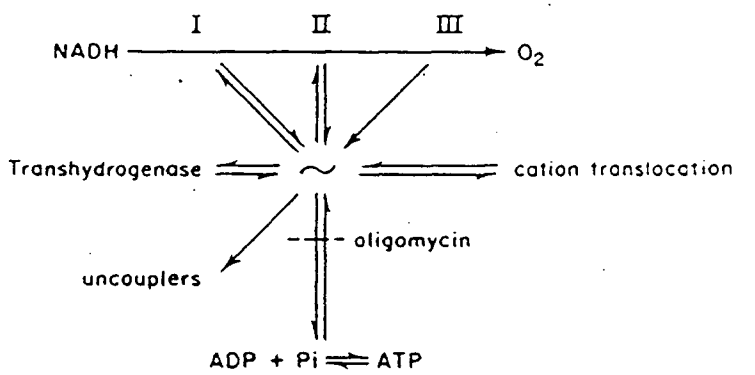
respiratory chain was proposed to drive reverse electron transport. Uncouplers were postulated to act by hydrolyzing the x~y compound. Some researchers even claimed to have isolated this high energy intermediate (26). Most of the cases were later proved to be based on faulty interpretation. Eventually, this "chemical coupling" hypothesis had to be abandoned.

But the idea of a high energy intermediate did not fade away and in 1965 Boyer (5) suggested that the conformations of proteins may change during oxidation and reduction and that similar conformational changes may occur in the ATP synthase. He proposed that conformational changes in the respiratory chain components during oxidation and reduction were transmitted directly to a closely located ATP synthase and that the resultant "strain" induced in the latter provided energy for ATP synthesis. The evidence supporting this hypothesis has been at best circumstantial. But despite the lack of supporting evidence, the concept of protein conformational changes has gained some support more recently as a possible mechanism for proton translocation in the chemiosmotic theory.

2. The Chemiosmotic Theory

In spite of the popularity of the chemical coupling and conformational hypotheses, the theory that has dominated the bioenergetics field since its introduction in 1961, is the Chemiosmotic theory proposed by Peter Mitchell (6). This theory denounced the existence of any high energy compounds and suggested instead a protonmotive force (fig. 1). The essence of Mitchell's proposal as applied to mitochondria is that: (a) the respiratory chain is asymmetrically organized in the mitochondrial inner membrane such that protons are vectorially translocated from the inside to the outside of the mitochondria as electrons are passed from substrate to oxygen; (b) the inner mitochondrial membrane is essentially impermeable to most ions, including both OH^- and H^+. Consequently, the electrogenic pumping of protons

a. Chemical hypothesis



b. Chemiosmotic hypothesis

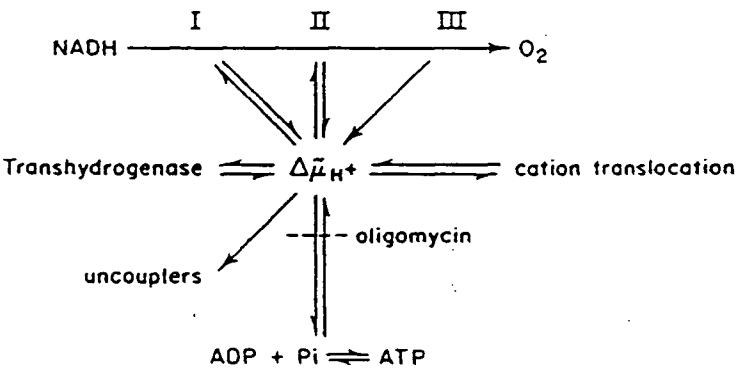


Fig. 1: Schemes of mitochondrial energy transduction

Mechanisms of membrane energization for mitochondria according to (a) chemical hypothesis and (b) chemiosmotic hypothesis. [~] represents an unknown 'high energy' intermediate. Figure reproduced from Nicholls, 1982 (55).

across the membrane creates an electrochemical gradient ($\Delta\mu_H$) which consists of both a pH gradient (ΔpH) and an electrical potential ($\Delta\psi$):

$$\Delta\mu_H = \Delta\psi - Z\,\Delta pH$$

where Z=2.303RT/F and serves to convert ΔpH into electrical units; (c) the electrochemical potential produced can then be used to drive the synthesis of ATP, translocate ions, perform reverse electron transport, drive energy-linked transhydrogenase reactions, or other forms of active transport processes. An analogous explanation is postulated for oxidative phosphorylation in bacteria and photophosphorylation in chloroplasts and photosynthetic bacteria.

At first, Mitchell's theory was not widely accepted, but a large flood of evidence soon poured in to support his hypothesis. It is briefly summarized here:

1. Respiration and ATP hydrolysis can generate an electrochemical proton gradient. Direct measurements of proton translocation by energy transduction systems were first reported in 1964 by Neumann and Jagendorf (7) for chloroplasts and by Mitchell in 1965 (8) with mitochondria.
2. An electrochemical gradient will lead to ATP synthesis. Jagendorf and Uribe (9) showed in 1966 that when an artificial $\Delta\mu_H$ is imposed on chloroplasts, ATP can be synthesized. Furthermore, Racker and Stoeckenius (10) used $\Delta\mu_H$ generated by illuminating bacteriorhodopsin in liposomes containing ATP synthase and bacteriorhodopsin to drive ATP synthesis.
3. The electrochemical gradient was shown to be a kinetically competent intermediate in oxidative phosphorylation by Thayer and Hinkle (11). They reported that ATP synthesis by an artificially imposed $\Delta\mu_H$ in submitochondrial particles is as fast or faster than the rate of ATP synthesis by respiration.
4. Uncouplers may act by dissipating the proton gradient (12).

The chemiosmotic theory reached its height of popularity by the late 1970's. Since then, evidence has been accumulating which argues against Mitchell's theory as originally put forward. A few of the more impressive data will be presented here:

1. Uncoupler-resistant mutants exist. One such example is *Bacillus megaterium* which can synthesize ATP even when $\Delta\mu_H$ is abolished by uncouplers (13).
2. $\Delta\mu_H$ can be very low in alkalophilic (14) and halophilic (15) bacteria under conditions in which they can synthesize ATP. For instance *Bacillus alkalophilus*, when grown with an external pH of 11, has only a Δp of 15 mV and yet it is still able to synthesize ATP.
3. ATP synthesis can be abolished without affecting $\Delta\mu_H$ by a group of substances known as decouplers (16).
4. Open membrane fragments of rabbit skeletal muscle have been proposed to be capable of energy transduction (17), when they should be structurally incompetent according to the chemiosmotic theory which requires enclosed membrane vesicles.

These discrepancies have dramatically lessened the attractiveness of the chemiosmotic theory. Many workers in the field have tried to search for an alternative explanation. Some have taken refuge in the belief that localized protons are the intermediates.

3. Localized Proton Theories

At about the same time that Mitchell proposed his chemiosmotic hypothesis, Williams (18) made an independent proposal that also involved protons as the intermediate in oxidative phosphorylation. He suggested that protons produced as a result of electron transfer are delivered directly to the ATP synthase where they promote the condensation of inorganic phosphate and ADP to form ATP. In this formulation, the protons remained in the membrane phase (fig. 2). It differs from

Mitchell's hypothesis in that a transmembrane potential is not necessary for ATP synthesis. In fact, the appearance of a bulk proton gradient is envisioned as a "leak" that is counter-productive to the energization of the membrane. Williams has had to modify his theory several times as new facts emerged. One of his most recent modifications is that direct proton binding on ATP synthase acts to drive two conformational changes (19). One releases ATP from F_1, the other opens the F_o channel to the inside of the mitochondria, allowing downhill diffusion of protons. This modification was made in light of the finding that the energy-requiring reaction in ATP synthesis is the dissociation of ATP and not the esterification of ADP.

Several other interesting "localized proton" theories have also been proposed by other researchers. Kell (20) suggested a 'protoneural' network of proteinaceous components that conducts protons between various protonmotive sources and proton accepting sinks by allowing rapid movement of protons along the surfaces of energy-transducing membranes. Along the same line of thought, Prats et al. (21) postulated the polar head groups of phospholipids at the surface of membranes to act as pathways for rapid proton conduction. This might occur by 'proton hopping' via a Grotthus mechanism (22). Westerhoff and colleagues (23) put forward the model of "mosaic chemiosmosis". They envisioned a mitochondrial membrane with different compartments for protons, all in communication with the bulk phase, but through a barrier of significant resistance. The protons are still translocated by a chemiosmotic mechanism but in small independently operating coupling units consisting of several respiratory chain assemblies associated with a limited number of ATP synthase molecules.

Using a completely different approach to this problem, Malpress (24) proposed a "coulombic hypothesis" where the primary mediating force in ATP synthesis is the electrostatic interaction generated between protons and fixed negative charges in selected, localized areas on the surface of the energized membrane. This is similar

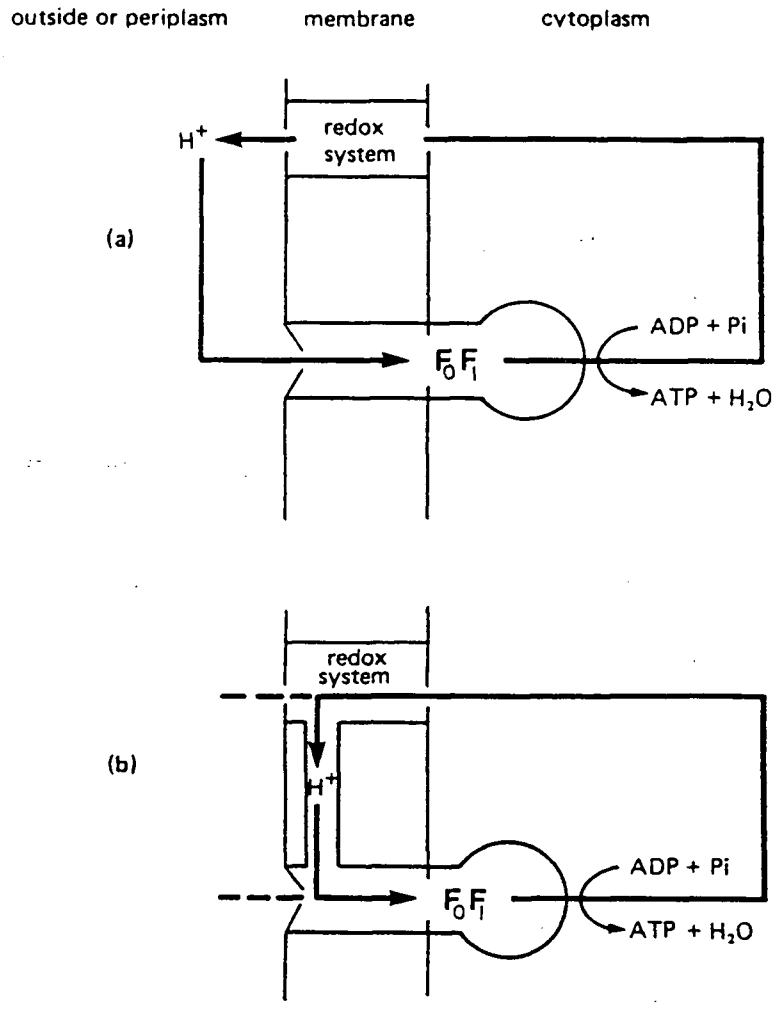


Figure 2: Bulk and localized proton circuits

Schematic diagram of the proton ciruit according to (a) the chemiosmotic hypothesis, and (b) the localized proton hypothesis. F_oF_1 symbolizes the ATPase. The dotted line in (b) represents a proton 'leak' to the bulk gradient. Figure modified from Jones, 1982 (57).

to the "local electric field" hypothesis put forward earlier by Skulachev (25). He considered the concept of local electric fields arising due to charge separation in a molecule of $\Delta\mu_H$-generating enzyme in affecting the adjacent $\Delta\mu_H$-utilizing enzyme such as ATP synthase.

4. Collision Hypothesis

In 1985, Slater and colleagues (26) postulated what they termed as the "collision hypothesis". They suggested that collision between freely diffusable energized redox enzymes and the ATP synthase is the manner by which energy is transferred between these two systems. This proposal may be written as:

$$A_{red} + B_{ox} + E_r \rightleftharpoons A_{ox} + B_{red} + E_r{\sim}h^+ \rightleftharpoons E_r + \Delta\mu_H \qquad (1)$$

$$E_r{\sim}h^+ + E_{ATP} \rightleftharpoons E_r + E_{ATP}{\sim}h^+ \rightleftharpoons E_{ATP} + \Delta\mu_H \qquad (2)$$

$$E_{ATP}{\sim}h^+ + ADP + P_i \rightleftharpoons E_{ATP} + ATP \qquad (3)$$

Reaction 1 describes a redox reaction, the end product of which is the 'high energy' conformation of the redox enzyme ($E_r{\sim}h^+$). This energy can be utilized either to create an electrochemical gradient ($\Delta\mu_H$) or can be transferred to the ATP synthase to form an energized form of the synthase ($E_{ATP}{\sim}h^+$) as shown by reaction 2. Subsequently, the energized synthase can use its energy to synthesize ATP or create $\Delta\mu_H$ (reaction 3). So similar to chemiosmosis, the redox enzymes and ATP synthase can still behave as proton pumps that deliver protons to the bulk phase. But the major route of energization is the transfer of energy via direct collision between the two molecules. A hypothesis almost identical to this one was independently proposed by Boyer (27) at about the same time.

Both their theories implicated the action of protonophoric uncouplers to be the direct interference between the redox enzymes and ATP synthase. Herweijer et al. (28) showed that the concentration of uncouplers needed is related only to the concentration of the interacting enzymes concerned with energy transduction. Evidence for this is found in the case of the uncoupler S13. Herweijer and colleagues demonstrated that low concentrations of S13 affected the direct energy transfer whereas high concentrations exhibited the classical mode of chemiosmotic uncoupling. That is, S13 uncoupled by behaving as a proton shuttling system.

B. The Electron Transport Chain

1. *E. coli* Respiratory Chain

To fully appreciate how the mechanism of energy coupling might operate, one must have some understanding of the components which make up the energy transducing membrane. One of the major assemblies in this membrane is the respiratory chain.

The respiratory chains of *E. coli* and mitochondria are very similar in their overall arrangement. In both, substrates are oxidized by dehydrogenases which are often flavoproteins associated with iron-sulfur centers. The dehydrogenases reduce quinones which in turn transfer electrons to cytochromes. The cytochromes or complexes containing cytochromes then transfer electrons to the terminal electron acceptor such as oxygen (29-30).

In contrast to the one major respiratory pathway of mitochondria, *E. coli* can synthesize a variety of respiratory chains depending on the conditions. Thus *E. coli* has the ability to induce several different dehydrogenases, each oxidizing specific substrates which may be NADH, formate, lactate, succinate, or α-glycerophosphate. The reduced dehydrogenases may subsequently reduce one or both species of ubiquinone-8 and menaquinone-8 (33). The reduced quinones in turn can terminate

in two different pathways under aerobic conditions. Under anaerobic conditions, the terminal electron acceptor may be nitrate, fumarate, or trimethylamine-N-oxide (TMAO) (29-32). Though these pathways are widely branched, there appears to be some favoured interactions between certain electron donors and specific electron acceptors. For instance, formate tends to donate its electrons to nitrate reductase while NADH is the preferred donor of electron to oxygen.

Since the present study involves only the aerobic respiratory chain of *E. coli*, this will be described in more detail.

2. Aerobic Electron Transport

There are two aerobic respiratory pathways in *E. coli* ; one terminating with cytochrome o and the other with cytochrome d. Both may, and often do, exist simultaneously under aerobic growth conditions. They share the same NADH dehydrogenases and both involve ubiquinone-8. They differ from each other at the level of the cytochromes (29).

Two immunologically distinct NADH dehydrogenases are found in the cytoplasmic membrane of *E. coli* (34). Matsushita et al. (35) differentiated the two enzymes by demonstrating that one enzyme system oxidizes both NADH and deamino NADH (dNADH), involves [4 Fe-4 S] and [2 Fe-2 S] type iron sulfur clusters, and is coupled to the generation of an electrochemical proton gradient. This is called NADH dh I. The other enzyme system, named NADH dh II, oxidizes NADH exclusively, has no iron-sulfur clusters, and does not generate a $\Delta\mu_H$. Hayashi et al. (36) further characterized the systems by showing that NADH dh I contains flavin mononucleotide (FMN) as a cofactor and catalyzes the reduction of ferricyanide. On the other hand, NADH dh II contains flavin adenine dinucleotide (FAD) as a cofactor and catalyzes the reduction of ubiquinone-1 (Q_1). Their ability to selectively reduce

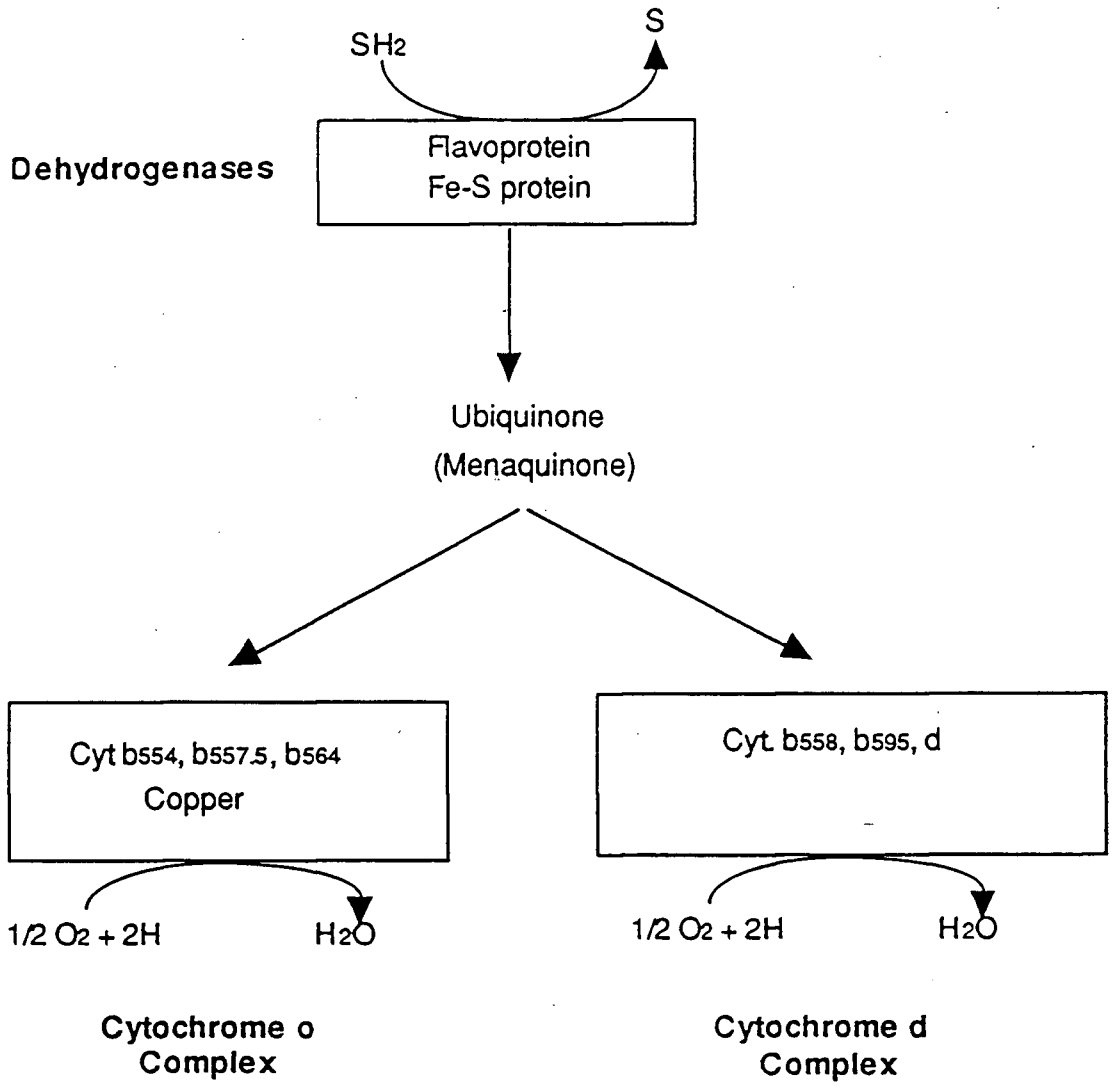


Figure 3: *E. coli* Respiratory Chain

Proposed arrangement of components in the respiratory chain of *E. coli*. No specific order is implied for the components listed in the boxes for dehydrogenase, cytochrome o and d complex. Fe-S, iron sulfur centre; cyt, cytochrome; S, Substrates.

either ferricyanide or Q_1 has formed the basis for enzymatic assays to distinguish between them.

E. coli can synthesize both ubiquinone-8 and menaquinone-8, although cells grown aerobically generally have a higher concentration of ubiquinone-8 than menaquinone-8. The converse is true under anaerobic growth conditions (37). However when the activity of the aerobic respiratory chain is impaired such as in heme deficient mutants, menaquinone is synthesized in high concentrations (38). It is not understood how the synthesis of the two quinones is regulated, but it is known that under some circumstances, they can substitute for each other to maintain electron transport activity (39).

When *E. coli* is grown under vigorous aeration, a set of redox carriers that involve ubiquinone-8 and the cytochrome o complex are induced. The latter consists of the cytochromes $b_{554\ (555)}$, $b_{564\ (563)}$ and probably $b_{557.5}$ (91). Which one of these cytochromes contains the oxygen reacting heme for the complex (cytochrome o) is not yet known. However, we know this heme reacts with carbon monoxide and is sensitive to cyanide. Kita and Anraku (93) have proposed this heme to be the one responsible for the peak at 555 nm whereas Withers and Bragg (91) argue that it is the heme which exhibits an absorbance peak at 564 nm.

The cytochrome d pathway is induced under conditions of low aeration. Cytochrome d differs from cytochrome o in that it has different spectral properties and is much less sensitive to inhibition by cyanide. Moreover, cytochrome b_{558} and b_{595} are associated with cytochrome d. Cytochrome b_{558} has been suggested to reduce cytochrome d but the role of cytochrome b_{595}, however, still remains a mystery (29). In addition to induction under low oxygen tension, the cytochrome d pathway has also been observed in a variety of apparently unrelated growth conditions such as aerobic growth in the presence of cyanide (41) or with limiting concentrations of sulfate (42).

Both of these pathways are usually present in cells grown aerobically. Figure 3 gives a general sequential order of electron transfer in both these pathways.

C. Pyridine Nucleotide Transhydrogenase

Pyridine nucleotide transhydrogenase is an integral enzyme found in the cytoplasmic membrane of *E. coli* and in the inner membrane of mitochondria. It catalyzes a reversible and direct transfer of a hydride ion equivalent between oxidized and reduced forms of NAD(H) and NADP(H) (44, 45). The transhydrogenase reaction is coupled to proton translocation across native mitochondrial membranes according to the following reaction (46):

$$NADPH + NAD^+ + n\,H_{matrix} \rightleftharpoons NADP^+ + NADH + n\,H_{cytosol}$$

where n is the number of protons translocated per hydride transferred. Values ranging from 0.2 - 2 have been determined for n, though no definitive value has yet been agreed upon (47). Kinetic data indicates the enzyme follows a random ternary-complex mechanism (95).

The transhydrogenase reaction can be driven by other energy transfer systems in the membrane such as the respiratory chain and the ATPase. These 'energy-dependent' transhydrogenase reactions produce a 5-10 fold increase in the rate (48) and a 500 fold increase in the equilibrium constant for the reduction of $NADP^+$ by NADH (49).

Bovine heart mitochondrial transhydrogenase has been purified to homogeneity employing methods that involved affinity chromatography on immobilized NAD^+ (96) or $NADP^+$ (97) and fast protein liquid chromatography (98). The *E. coli* transhydrogenase has also been purified by pre-extraction of the cytoplasmic membrane with sodium cholate and Triton X-100 followed by

solubilization of the enzyme with sodium deoxycholate in the presence of 1 M potassium chloride and centrifugation through a discontinuous sucrose gradient (99). Reconstitution studies of both mitochondrial and *E. coli* enzymes have confirmed that it is a proton pump, using 9-aminoacrine as a pH probe (99, 100).

Structurally, transhydrogenase is a relatively simple enzyme. Its amino acid sequences from bovine mitochondria (50) and *E. coli* (51) have been deduced via gene cloning and sequencing. The mitochondrial transhydrogenase was found to be a homodimer whose monomers have a M_r=110,000. In contrast, the *E. coli* enzyme is composed of two types subunits α (M_r=54,000) and β (M_r=48,700), and exists as an $\alpha_2\beta_2$ tetramer (94). The NAD(H) and NADP(H) binding sites are believed to be near the N and C termini respectively of the bovine mitochondrial enzyme (52). These domains correspond to the N terminal region of the α and the C terminal region of the β subunit of *E. coli* transhydrogenase respectively. Hydropathy plots predict as many as 14 transmembrane regions for the mitochondrial transhydrogenase (50) and 12 for the *E. coli* transhydrogenase (72).

A number of other facts are also known about the enzyme. For example, cysteine residues are believed to be involved in its catalytic activity (53). The enzyme can undergo large, energy-induced conformational changes during catalysis (54). DCCD inactivates both the proton translocating and catalytic activity of the enzyme (101). Magnesium inhibits the enzyme (102). Yet despite all these data, the physiological significance of this enzyme is still unclear.

D. Objective of this thesis

The structural simplicity of the transhydrogenase, the existence of convenient real time assays, together with its unique relationship to other energy transducing systems has made this enzyme an attractive system by which to study the mechanism of membrane energization. Indeed, Slater (3) had interpreted the energy-dependent

transhydrogenase reaction in terms of using a non-phosphorylated high energy intermediate during the era of the "chemical" hypothesis. This was reinterpreted a decade later in favour of the chemiosmotic theory by Mitchell (55) as shown in figure 1. The work from this thesis shall present some evidence for the "collision hypothesis".

Prior to the commencement of this project, Clarke and Bragg (56) had cloned the *E. coli* transhydrogenase gene into a multicopy vector pUC 13 after screening for clones in the Clarke and Carbon colony bank (56) that showed elevated levels of transhydrogenase activity. The recombinant plasmid was termed pDC21. The plasmid was then transformed into the *E. coli* JM83 host to create the transformant JM83pDC21F$^-$. This transformant showed a 70 fold increase in transhydrogenase activity in comparison to the host strain.

This thesis will describe the pattern of energization of the transhydrogenase enzyme by itself and under conditions whereby it is energized by other systems (i.e. electron transport chain or ATPase). The strategy pursued was one where the enzymatic and proton translocating activities were measured with a wide variety of uncouplers and ionophores. Both the parent and transformed strains of JM83 and W6 were compared for their mode of energization. Furthermore, the respiratory chains of these strains were investigated spectrometrically in an attempt to detect any differences between the parent and transformed strains. Finally, a partial separation of the transhydrogenase containing vesicles from the vesicles containing respiratory chain components was demonstrated for JM83pDC21F$^-$.

II MATERIALS AND METHODS

A. Materials

All chemicals were obtained from commercial sources and were either of reagent or analytical grade. Special chemicals were purchased from the following suppliers:

DIFCO	-Bactotryptone, agar, and yeast extract
SIGMA	-NAD(H), NADP(H), 3Ac NAD(H), yeast alcohol dehydrogenase, ATP, QNO, DCCD, deamino NADH, ampicillin, CCCP, valinomycin, nigericin, PMS
BIORAD	-All polyacrylamide gel electrophoresis reagents and molecular weight standards, alkaline phosphatase conjugated goat anti-rabbit IgG, nitrocellulose membrane
Nutri. Biochem. Co.	-Quinacrine (Atebrin)
BDH	-Hydrogen peroxide, sodium dithionite, ascorbate
FISHER	-Potassium ferricyanide, potassium cyanide
Promega Biotec	-(BCIP/NBT) 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium alkaline phosphatase development system
Eastman	-TCS
J.T. Baker Chem.	-Thiamine
Alfa products	-TBTC, TPTC

B. Growth of Bacterial Strains

The *E. coli* strains of JM83pDC21F$^-$ *ara Δ lac pro str A thi φ80d lac Z ΔM15*, W6pDC21F$^-$ *pro* and their respective parent strains of JM83 and W6 were used in this study. These strains were stored at -50 °to -70 °C in 25% glycerol.

These cells were grown in M9 medium: 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.05% sodium citrate, 0.02% $MgSO_4 \cdot 7H_2O$, 0.1% $(NH_4)_2SO_4$, 1 μg/ml thiamine, 50 μg/ml

proline, 12 μM Ferric citrate, and 0.4% glucose. The plasmid strains (i.e. pDC21F$^-$) were selected for by addition of 100 μg/ml ampicillin into the media.

To facilitate growth, the cultures were incubated at 37 °C and shaken at 250 rpm until mid-log phase was reached. They were harvested by centrifugation at 4,400 x g for 20 minutes. The pellets were washed with 0.9% NaCl followed by centrifugation at 12,000 x g for 10 minutes, twice. The resulting cell pellets were either used immediately or stored at -50 °C.

C. Isolation of Cell Membranes

All steps were performed at 0-4 °C. The cell pellets were first suspended at 1 g / 5 ml (wet weight) in membrane buffer (50 mM Tris-H_2SO_4, 5 mM $MgSO_4 \cdot 7H_20$ 1 mM DTT, 1 mM EDTA, 1 mM DNase I). They were then lysed by passage through an ice-cold AMINCO French Press at 20,000 psi, twice. This made everted membrane vesicles. Unbroken cells and debris were removed by centrifugation at 12,000 x g for 10 minutes. The resulting supernatant was centrifuged at 180,000 x g for 2 hours to pellet the membrane and remove most of the soluble proteins. The final membrane pellet was suspended at 1 g membranes / 5 ml membrane buffer and kept at -50 °C or used immediately.

D. Determination of Protein Concentration

Protein concentration was determined by the method of Lowry (58) over a concentration range of 0-500 μg/ml. Bovine serum albumin was used as the standard and the Folin-Ciocalteau reagent was diluted 1:1. The absorbance readings were taken at 660 nm.

E. Enzymatic Assays

1. Energy-Independent Transhydrogenase Assays

This assay of transhydrogenase activity is based on the method of Kaplan (59). An appropriate sample of membrane vesicles was suspended in 1 ml of assay buffer (50 mM Tris-H_2SO_4 pH 7.8, 5 mM $MgCl_2 \cdot 6H_2O$, 2 mM DTT, 300 mM KCl) at room temperature followed by addition of substrates to 1 mM AcNAD and 0.5 mM NADPH, final concentrations. The reduction of AcNAD by NADPH was measured as an absorbance increase at 375 nm using a Perkin-Elmer Lamda 3A spectrophotometer. The extinction coefficient for AcNADH is 5.1 l/mmol/cm. One unit of enzyme activity represents the conversion of 1 µmol of AcNAD to AcNADH per minute.

2. Energy-Dependent Transhydrogenase Assays

This assay is a modification of the method of Fisher and Sanadai (60). As before, an appropriate sample of membrane vesicles was suspended in 1 ml of assay buffer. 50 µl of yeast alcohol dehydrogenase (4 mg/ml), 5 µl of ethanol, and 25 µl of 3 mM NAD^+ was then added. The absorbance of the reaction mixture was followed at 340 nm. The initial decrease in absorbance is due to NADH oxidase activity. After 2 minutes, 25 µl of 16.3 mM $NADP^+$ was added and the aerobic-driven transhydrogenase activity was measured. When the oxygen in the cuvette was exhausted, the slight amount of NADPH formation was now due to energy-independent transhydrogenation. 10 µl of 60 mM ATP was then added and the ATP-driven transhydrogenase activity measured. The rate of the energy-driven transhydrogenation (aerobic and ATP) was corrected by subtracting the rate of energy-independent transhydrogenation. The extinction coefficient for NADPH is 6.22 l/mmol/cm. One unit of activity represents reduction of 1 µmol of $NADP^+$ to NADPH.

3. Fluorescence Assays

Intravesicular pH changes were followed fluorometrically with a Turner spectrofluorometer (model 430) using the fluorescent probe quinacrine at the indicated wavelength pair (excitation 430 nm; emission 505 nm). The assay buffer consisted of 10 mM Hepes-KOH (pH 7.4), 5 mM $MgCl_2$, 2 mM DTT, and 300 mM KCl. First, an appropriate sample of membrane vesicles together with quinacrine to 5 mM was added to 1.9 ml of the assay buffer. For the assay of proton translocation accompanying transhydrogenation from NADPH to 3AcNAD, the reaction mixture contained 1 mM 3AcNAD and 0.5 mM NADPH. For the assay of proton translocation associated with NADH oxidation or ATP hydrolysis, the mixture contained 0.5 mM NADH and 0.6 mM ATP, respectively.

4. Ferricyanide and Q_1 reductase assays

This assay is based on the method of Kaback et al. (61) and modified accordingly to suit experimental needs. The reaction mixture contained an appropriate amount of membranes, 20 mM KCN, and a particular amount of an electron acceptor in 1 ml of 50 mM Tris-H_2SO_4 (pH 7.8)/5 mM $MgSO_4$. Reactions were started by addition of 200 μM NADH or d-NADH as indicated. Q_1 reductase activity was measured by the change in absorbance at 340 nm with 200 μM Q_1. Ferricyanide reductase activity was measured by the change in absorbance at 420 nm with 1 mM ferricyanide. Activities were calculated by using millimolar extinction coefficients of 6.81 and 1.0 for Q_1 and ferricyanide, respectively. Assays were carried out at ambient temperature.

5. NADH, d-NADH, and PMS/Ascorbate Oxidase Assays

NADH and d-NADH oxidase activity was measured by two separate methods. The first involved following the activity spectrometrically at 340 nm using the

Perkin Elmer Lamda 3A spectrophotometer. The assay buffer contained 50 mM Tris-H_2SO_4 (pH 7.4), 5 mM $MgSO_4 \cdot 7H_2O$, 2 mM DTT, and 300 mM KCl. To start the reaction, NADH or d-NADH was added to a final concentration of 200 μM in 1 ml of assay buffer containing membranes. The extinction coefficient was taken to be 6.22 l/mmol/cm.

The second method involved the use of the Dual Wavelength SLM Aminco 2C spectrophotometer with the band width set at 2.2 nm. The difference in absorbance at 559 nm relative to 580 nm was measured. The same assay buffer as in the first method was used. The reaction was started by addition of NADH or d-NADH to a final concentration of 5 mM in 2 ml of assay buffer with membranes. The activity was measured from the time taken to deplete oxygen from the solution, as determined by cytochrome reduction at anaerobiosis, and calculated as μg atoms O_2 consumed/min/mg protein (assuming a concentration of 0.52 μg atoms O_2/ml assay buffer). PMS/ascorbate oxidase activity was measured using only the latter method. 2.5 μM PMS and 2 mM ascorbate was added to start the reaction.

F. Measurement of Cytochrome Spectra

Cytochromes were measured via dithionite reduced *minus* oxidized difference spectra at 77 K with a SLM-Aminco DW2C double beam spectrophotometer. 3 separate spectra for each sample were collected and averaged. A band width of 2.2 nm was used for the range of 400-700 nm. For detailed examination of the alpha band region, spectra were scanned between 515 and 590 nm using a band width of 0.8 nm. Fourth-order finite difference spectra were obtained by processing data with the Midan II kinetics processor/controller of the spectrophotometer.

G. Quinone Extraction

This method is a modification of the technique used by Klingenberg et al. (62). 0.5 ml of membrane suspension corresponding to 0.5-2 mg protein was first thoroughly mixed with 2.5 ml of methanol and petroleum ether (60:40 v/v). 1 ml of acetone was then added to the mixture which was then stirred for 15 minutes. The extracted membrane was subsequently pelleted by centrifugation at 3000 x g for 5 minutes. The upper lighter petroleum layer was transferred to a separate tube. The residue was reextracted with 1 ml of petroleum ether and its upper layer was withdrawn and added to the solution in the tube. The combined extracts were evaporated at about 35 °C under reduced pressure using a rotary evaporator. The dry residue was dissolved in 2 ml of ethanol. To measure the amount of quinone, spectra were run between 400 and 200 nm. The difference in absorption between the spectra at 275 nm, before and after reduction with KBH_4, was used to calculate the concentration assuming $\Delta E = 8.8\ mM^{-1}cm^{-1}$.

H. SDS Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis, based on the method of Laemmli (63), was performed using a Bio-Rad Mini-Protean II gel apparatus according to the manufacturer's instructions. The gels were 7 cm in length and 0.75 mm thick. The separating gel was poured as a 10% gel containing 0.375 M Tris-HCl (pH 8.8), 0.1% SDS, and 7.5% acrylamide (prepared from a stock solution of 30% (w/v) acrylamide and 0.8% (w/v) N,N'-methylenebisacrylamide in water). Polymerization was effected by addition of 200 µl of freshly made 10% Ammonium persulfate and 14 µl of TEMED to 40 ml of gel. The stacking gel was poured as a 5% gel of 0.125 M Tris-HCl (pH 6.8), 0.1% SDS, and 4% acrylamide. Polymerization was initiated by adding 150 µl of 10% Ammonium persulfate and 15 µl of TEMED to 15 ml of gel. The samples were prepared by adding an equal volume of SDS sample buffer (0.125 M

Tris-HCl pH 6.8, 4% SDS, 10% β-mercaptoethanol, and 20% glycerol). No heating was required for the samples containing membrane vesicles. The running buffer was made up of 25 mM Tris base, 192 mM glycine, and 0.1% SDS. Electrophoresis was carried out at 200 V for 45-60 minutes at room temperature. Protein gels were then stained with Fairbank's stain (0.1% (w/v) Coomassie brilliant blue R, 25% (v/v) isopropanol and 10% (v/v) acetic acid) and destained with 10% (v/v) acetic acid. They were finally dried down at 80 °C for 1/2 - 1 hour under reduced pressure in a Bio-Rad model 224 gel slab dryer.

I. Immunoblots

Samples were subjected to SDS-polyacrylamide gel electrophoresis as described previously and then transferred onto blot-qualified nitrocellulose paper by electrophoretic elution (64) using a Bio-Rad Trans Blot cell according to the manufacturer's instructions. The transfer buffer used contained 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. Transfers were done either at 60 V, 210 mA for 5 hours or at 70 V, 250 mA for 3 hours.

The transferred proteins were immunostained as described by Harlow and Lane (65). The trans-blotted nitrocellulose membrane was initially treated with 5% fetal calf serum in KBS buffer (137 mM NaCl, 1.5 mM KH_2PO_4, 7.2 mM Na_2HPO_4, 0.02% NaN_3, 2.7 mM KCl, 0.05% Tween 20) for 60 minutes at room temperature or at 4 °C overnight to block non-specific antibody binding sites. It was then washed 3 times in KBS and incubated with the primary rabbit anti-transhydrogenase antibody for 30-60 minutes. Subsequently, it was washed 3 more times with KBS and incubated with the alkaline phosphatase conjugated secondary goat anti-rabbit antibody (diluted as instructed by the supplier) for 30-60 minutes at room temperature. The membrane was washed 3 more times in KBS and equilibrated in alkaline phosphatase substrate buffer (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM

$MgCl_2$) for 5 minutes. Development of the immunoblot was achieved by addition of 22 µl nitro blue tetrazolium and 16.5 µl 5-bromo-4-chloro-3-indoyl phosphate to the nitrocellulose membrane in 5 ml of alkaline phosphatase substrate buffer for 10-15 minutes. The reaction was terminated by washing the membrane with water.

J. Plasmid Isolation and Transformation of Competent cells

1. Mini plasmid preparation

Small scale preparations of plasmid DNA were done using the alkaline lysis method as described by Meniatis et al. (66). The desired bacterial strain was first grown overnight at 37°C in 5 ml of penassay broth. 1.5 ml of the culture was then poured into an eppendorf tube and centrifuged for 1 minute in a Micro-centrifuge. The medium was removed by aspiration and the cell pellet was resuspended in 100 µl of ice-cold lysis buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0). After 5 minutes of incubation at room temperature, 200 µl of freshly prepared ice cold solution of 0.2 N NaOH containing1% SDS was added to the suspension and the contents were carefully mixed by inverting the tube 2-3 times. 150 µl of an ice cold potassium acetate (pH 4.8) solution (60 ml of 5M potassium acetate, 11.5 ml of glacial acetic acid, and 28.5 ml of water) was then added and the resulting mixture was left on ice for 5 minutes. The solution was then centrifuged for 10 minutes in a micro-centrifuge and the supernatant transferred to a fresh tube and extracted with an equal volume (i.e. 450 µl) of phenol/chloroform (24 :1 v/v). This was followed by centrifugation for 15-30 seconds in a micro-centrifuge with the supernatant transferred once more to a fresh tube. Two volumes (i.e.1 ml) of ethanol were then added. Subsequently, it was left standing at room temperature for 15 minutes before centrifuging the tubes for 7 minutes. The supernatant was then removed and the pellet dried in a vacuum dessicator for approximately 5 minute. 50 µl of TE buffer (10 mM Tris, 1 mM EDTA) containing 50 µg/ml DNase-free pancreatic RNase was added

to dissolve the pellet and the resulting mixture was incubated at 37 °C for 5 minutes. The preparation of plasmid DNA was stored at -20 °C refrigeration until use.

2. Preparation of Competent cells

Competent cells were prepared by the calcium chloride procedure (66). An innoculum of the desired bacterial cells was grown overnight at 37 °C in 5 ml of penassay broth (PAB). 100 µl of this starter culture was then added to 35 ml of fresh PAB medium in a 500 ml flask. This was incublated at 37 °C with vigorous shaking to an absorbance at 600 nm of 0.2 to 0.6, at which time the cells were harvested by centrifugation at 3,000 x g for 5 minutes at 2 °C. These cell pellets were then suspended in 17.5 ml of ice cold 50 mM $CaCl_2$ and kept at 0 °C for 30 minutes before being pelleted again. The resulting supernatant was discarded and the pellet was gently resuspended in 2.5 ml of 50 mM $CaCl_2$. It was then kept at 0 °C for 12-24 hours prior to the transformation.

3. Transformation

Approximately 10 ng of plasmid DNA (usually 1-2 µl of plasmid preparation) was added to a 300 µl sample of competent cells. The mixture was kept at 0 °C for 30-40 minutes before being heat shocked for 2 minutes in a 42 °C water bath. The cells were then revived by addition of 0.7 ml penassay broth to each tube and grown at 37 °C with shaking for 1 hour. Up to 100 µl of the transformed cells were streaked onto agar plates with ampicillin (100 µg/ml). To ensure that transformation had occured, colonies were grown up and assayed for amplified enzymatic activity as expressed by the plasmid.

K. Membrane Fractionation

Bacterial cells were grown to mid-log phase, harvested, and ruptured by a French Press cell as previously mentioned. After removing the unbroken cells and debris by centrifugation at 12,000 x g for 10 minutes, 200 µl of the lysate (approximately 300 µg protein) was layered onto a 5 ml 20-45% (w/w) linear sucrose gradient (Note: The sucrose gradient was made up in membrane buffer) and centrifuged at 4 °C for 16 hours in a SW 50L Beckman rotor at 30,000 rpm. The gradient was fractionated by puncturing the bottom of the tube and collecting ten 0.5 ml fractions. These fractions were used directly for SDS polyacrylamide gel electrophoresis and enzymatic assays.

III RESULTS

A. Effects of various metals on energy-independent transhydrogenase activity

Lee and Ernster (104) have demonstrated that certain divalent metal ions inhibit the transhydrogenase activity in bovine heart submitochondrial particles. Mg^{2+}, in particular, has been shown to inhibit the energy-independent transhydrogenase reaction to a greater degree than the energy-dependent reactions, with the extent of inhibition increasing with increasing medium pH (105). O'Neal and colleagues (102) have reported Mg^{2+} to be a competitive inhibitor with respect to $NADP^+$, and is therefore presumably specific for the catalytic NADP(H) site.

No parallel set of experiments have been done on the *E. coli* system. The work in this thesis shows that, at low concentrations, group IIA metals such as Mg^{2+}, Ca^{2+} and Ba^{2+} stimulate energy-independent transhydrogenase activity rather than inhibit it (fig. 4). Mn^{2+}, which is a similar metal to Mg^{2+}, does not show as marked a stimulated effect. At high concentrations, Mg^{2+} inhibits the energy-independent transhydrogenase reaction, in a similar manner to the mitochondrial system (fig. 5). This inhibitory effect is not due to salt denaturation of the enzyme as shown by the control curve with NaCl.

At present, the nature of these effects are not understood. The fact that several group IIA metals stimulate energy-independent transhydrogenase activity implies that electronic effects can affect the transhydrogenase enzyme. Inhibition of transhydrogenase activity at high concentrations of Mg^{2+} has been suggested by O'Neal et al. (102) to occur via binding of Mg^{2+} to the $NADP^+$ site, resulting in conformational changes of the enzyme which inhibit its activity. Stimulation by low concentrations of Mg^{2+} may occur, other than by the electronic effect proposed above, by formation of a Mg^{2+} complex with the phosphate groups of NADPH. This

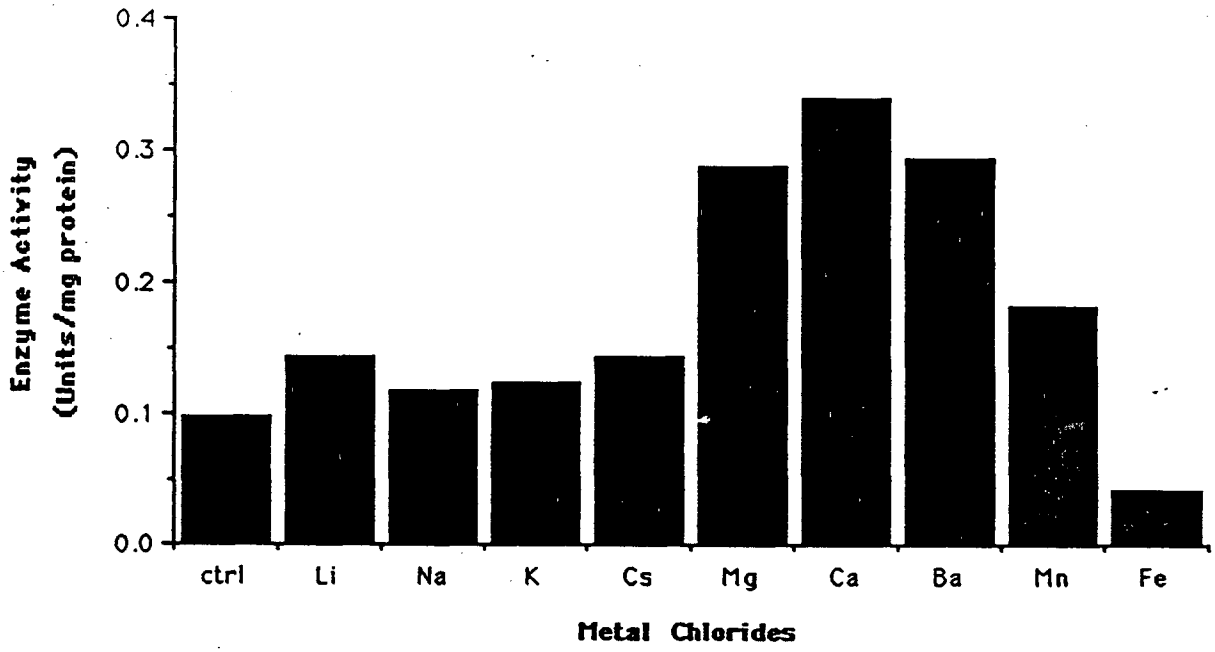


Fig. 4: Effect of metal ions on energy-independent transhydrogenase activity of strain JM83pDC21

Measurement of energy-independent transhydrogenase activity is as described in Materials and Methods with the exception that no magnesium is present in the assay buffer until indicated. The various metal chloride ions were added to a final concentration of 5 mM. 68 mg of protein was added to each assay mixture.

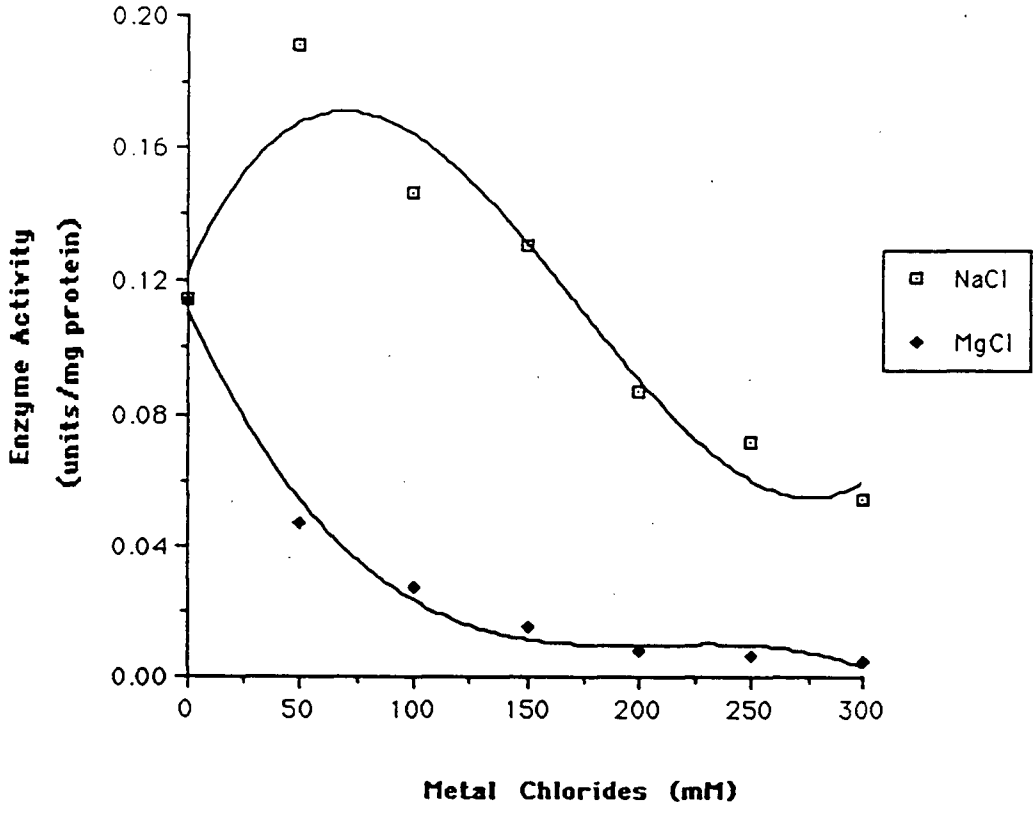


Fig. 5: Effect of high concentrations of Magnesium on energy-independent transhydrogenase activity of strain JM83pDC21

Measurement of energy-independent transhydrogenase activity is as described in Materials and Methods with the exception that no magnesium is present in the assay buffer until indicated. 68 mg of protein was added to each assay mixture. The effect of NaCl was measured as a control.

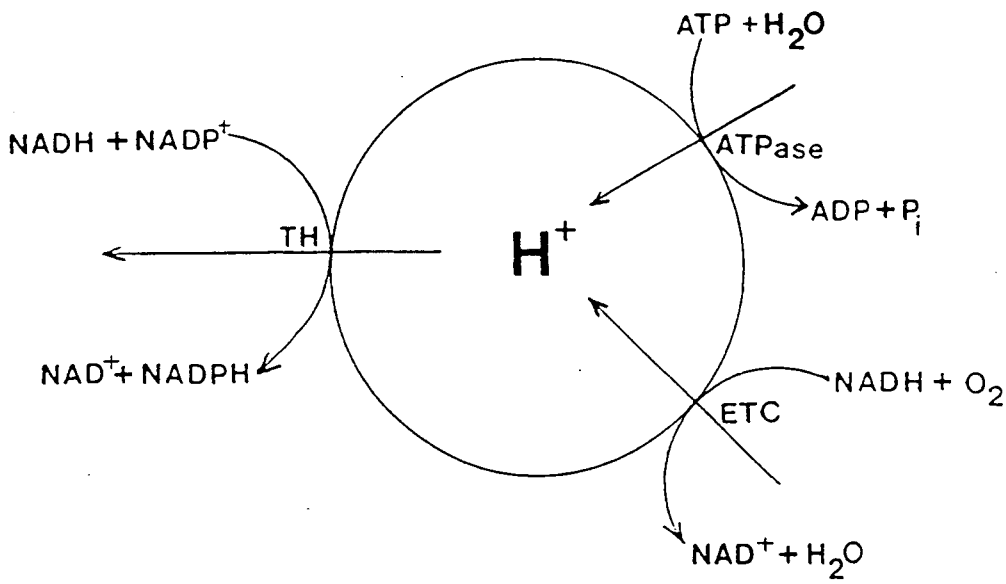
proposal draws analogy with many other glycolytic enzymes that require Mg^{2+} for activity. In these cases, Mg^{2+} forms a complex with the phosphate groups of the substrates such as ADP and ATP. These complexes bind to the substrate binding site of glycolytic enzymes which appear to be specific for the Mg^{2+} complex of the phosphorylated intermediate rather than the intermediate itself. All of the proposals above are speculative and lack direct evidence.

B. Energization of transhydrogenase and energy-dependent transhydrogenase reactions

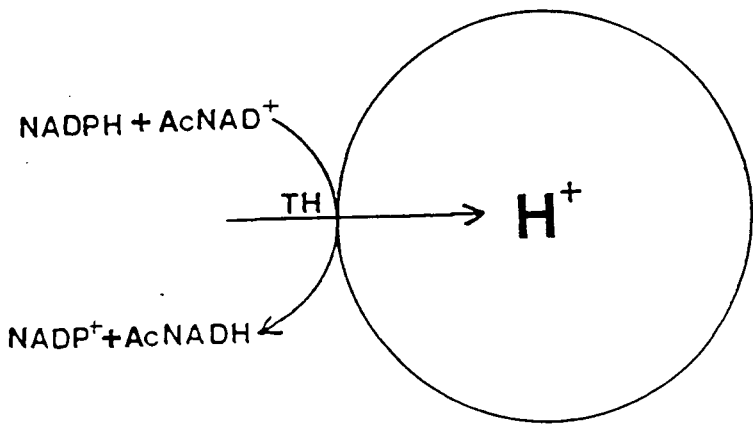
The different enzyme assays and their mode of energization, as proposed by the chemiosmotic theory, are schematically represented in fig. 6 and 7. The results henceforth, will be described in reference to these diagrams.

1. Energization of Transhydrogenase

Membrane vesicles containing transhydrogenase were isolated and measured for their energy-independent enzymatic activities in the presence of different agents as shown in fig. 8(a). The control value is the enzyme activity in the absence of agents and is designated to be 100%. Three major classes of agents were added to the enzyme assay system to investigate the mode of energization of the transhydrogenase. The first class is composed of the uncouplers TCS, CCCP, and valinomycin in the presence of nigericin. These compounds are postulated to function by collapsing any proton gradient that might be built up by the energy-transducing components of the membrane (67). The next class is the ionophores. As the name implies, these reagents transfer ions. Valinomycin allows the electrogenic flow of potassium ions down their concentration gradient and nigericin promotes the electroneutral exchange of potassium ions and protons (68). Both TBTC and TPTC catalyze the electroneutral exchange of chloride and hydroxide ions



a



b

Fig. 6: Diagram of the energy-independent and energy-dependent transhydrogenase assays

The enzyme assays and their coupled proton movement as predicted by the chemiosmotic theory are shown. (a) represents the energy-dependent assays of transhydrogenase activity energized by ATP hydrolysis and by substrate oxidation through the respiratory chain. (b) represents the energy-independent assay of transhydrogenase activity. TH, transhydrogenase; ETC, electron transport chain; H^+, indicates the vectorial translocation of protons. No stoichiometry is implied.

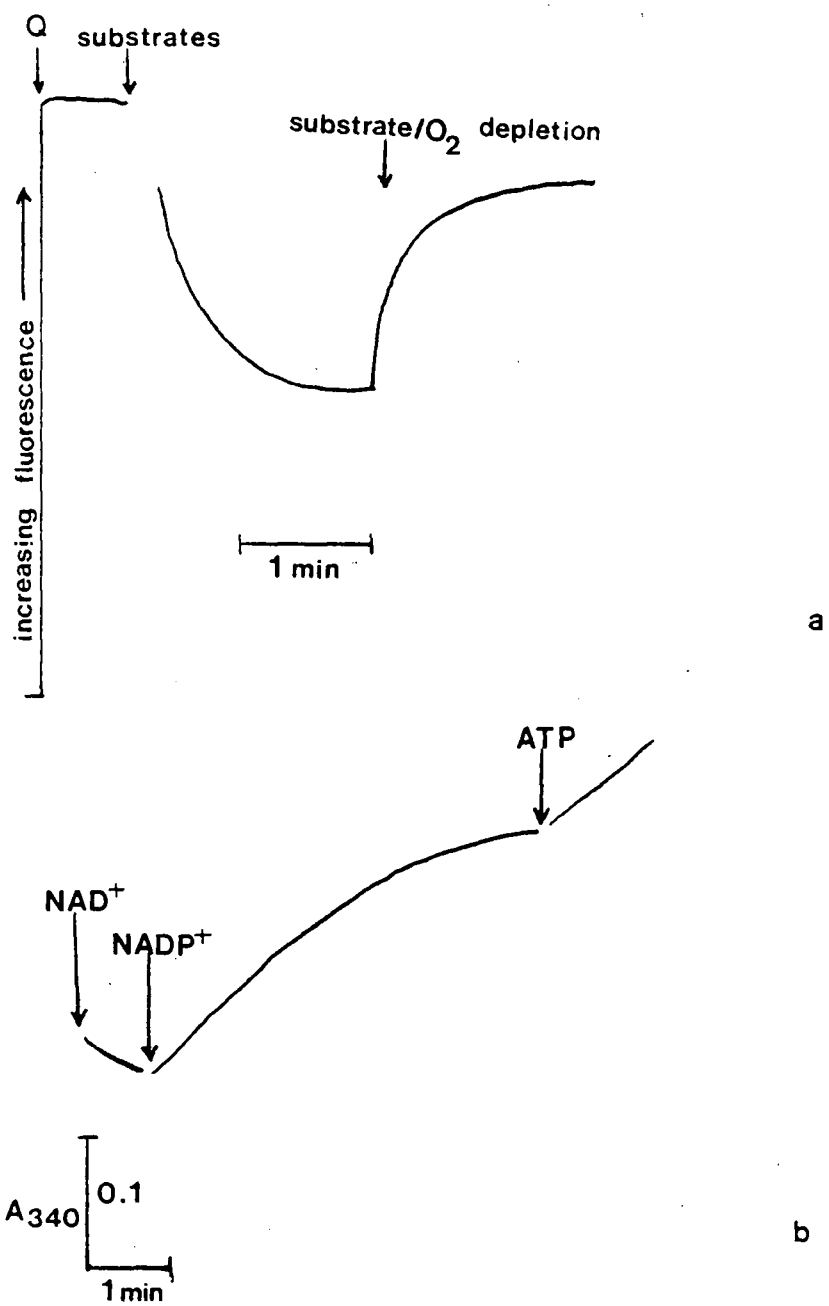


Fig. 7: Sample traces of quinacrine fluorescence and energy-dependent transhydrogenase assays

Proton translocation by fluorescence quenching (a) and and energy-dependent transhydrogenase assays (b) are run as described in Materials and Methods. These traces represent sample data from both these assays. Q, quinacrine.

with the latter compound being the more effective (69). The last class of agents are the respiratory chain inhibitors QNO and KCN (70). They were included as controls and their use will become significant, as will be explained later in the section dealing with aerobic-dependent activities. Proton movements may be monitored by various pH probes such as quinacrine, pyranine, or neutral red. In fig. 8(b), proton translocation during the reduction of AcNAD by NADPH was followed by measuring the fluorescence of quinacrine (71). Fluorescence quenching concurrent with transhydrogenase activity indicates uptake of protons into membrane vesicles during the enzymatic reaction.

Fig. 8(a) demonstrates, that in the presence of uncouplers, the rate of the energy-independent transhydrogenation of 3AcNAD by NADPH is increased 2-3 fold implying that a proton gradient is involved in the reaction. This is supported by the lack of fluorescence quenching upon uncoupler addition in fig. 8(b). The addition of valinomycin, however, shows a relatively small effect despite the imposition of a membrane potential in all these assays by the presence of an external 300 mM KCl gradient (see Materials and Methods). The presence of valinomycin should have generated a positive membrane potential inside the vesicle without affecting ΔpH, to inhibit both the enzymatic and fluorescence quenching activities. Its inability to do so in both cases (fig. 8), suggest that $\Delta\psi$ does not have a role in transhydrogenase energization. The stimulation by valinomycin in fig. 8(a) is atypical of the results from many other experiments (data not shown). Nigericin, on the other hand, affects only ΔpH and not $\Delta\psi$. Its addition should stimulate the enzymatic activity and dissipate fluorescent quenching under the assay conditions. Fig. 8 shows that this is indeed what happens. Therefore, ΔpH appears to be the more important component of the electrochemical gradient.

Since TBTC and TPTC exchange OH^- for Cl^- ions, in the presence of a high external KCl concentration, their addition should result in an increase of internal

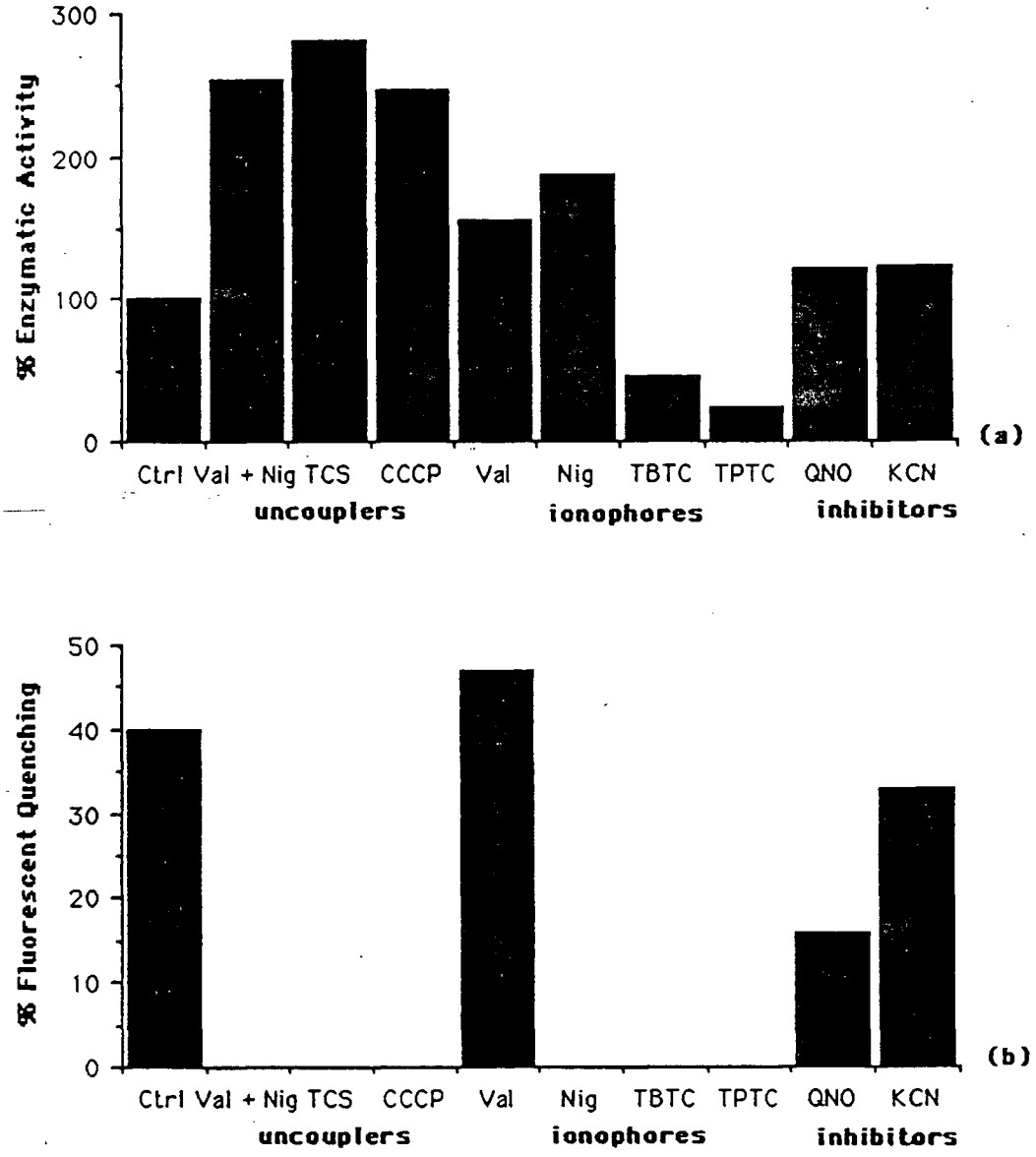


Fig. 8: Effect of uncouplers, ionophores, and respiratory chain inhibitors on energy-independent transhydrogenase activity and proton translocation in inverted membrane vesicles of strain JM83pDC21

Measurement of energy-independent transhydrogenase activity (a) and proton translocation by fluorescence quenching (b) are as described in Materials and Methods. 32 µg and 3.2 mg of protein was added to each assay mixture for (a) and (b) respectively. The agents of val+nig, TCS, CCCP, val, nig, TBTC, TPTC, QNO and KCN were added independently to the final concentrations of 4.5+7, 2, 10, 9, 14, 25, 25, 100 µM and 10 mM, respectively. The control (100%) value in (a) was 0.57 units/mg protein.

protons in the membrane vesicle causing an inhibition of the proton coupled enzymatic reaction and maintaining or stimulating the quenching of the fluorescent probe quinacrine. Fig. 8 shows that although the enzymatic activity was inhibited, fluorescence quenching was also inhibited. The significance of these results will be discussed later. Lastly, the respiratory chain inhibitors had little or no effect on the enzymatic energy-independent transhydrogenase activity. Furthermore, KCN addition did not affect fluorescence quenching though QNO addition had a slight effect (fig. 8b) indicating that the latter may behave as a weak uncoupler.

The above effects of various agents on energy-independent transhydrogenase assays was performed nine times with membranes from four strains of *E. coli* (JM83, JM83pDC21F$^-$, W6, and W6pDC21F$^-$) and a representative set of data is shown. They show that these effects were not strain specific. However, differences do exist between the plasmid containing strains and the non-plasmid containing strains with respect to their specific activity. The former have specific activities in the range of 0.5 units/mg protein whereas in the latter they are in the range of 0.05 units/mg protein. Furthermore, in some cases, the stimulation or inhibition of enzyme activity by various agents is better seen in the plasmid strains than the non-plasmid strains. These results are expected since the plasmid strains contain amplified levels of transhydrogenase enzymes in their membranes.

2. Energization of ATP-dependent transhydrogenase activity

As mentioned in the Introduction, transhydrogenase is a component of the energy transducing membrane that includes the ATPase and the respiratory chain. Its enzymatic activity can be driven by membrane energization via other energy sources such as ATP as shown by fig. 6 (73). A study of ATP-dependent transhydrogenase activity, exposed to the agents previously mentioned, can provide us with information regarding the energization mechanism linking these two systems.

Figure 9(a) shows the data pertaining to the ATP-dependent transhydrogenase activities. The control is the ATP-dependent activity in the absence of any agents and is designated as 100%. All three uncouplers inhibited ATP-dependent transhydrogenase activity, possibly by destroying the "energy-linking intermediate". The fluorescence assays of fig. 9(b) implicate this intermediate to be the proton gradient since uncoupler addition dissipates the proton quenching. Valinomycin had little or no effect on the transhydrogenase activity and on ATP-dependent fluorescence quenching, indirectly verifying the results of the energy-independent assays mentioned before. Nigericin inhibited the ATP-dependent transhydrogenase activity because it dissipated the ATP generated internal protons used to energize the transhydrogenase reaction (fig. 6). This hypothesis is supported by its ability to dissipate the fluorescence quenching as well (fig. 9b). It is significant that TBTC and TPTC inhibited both the transhydrogenase activity and fluorescence quenching. An explanation for their mechanism of action will be outlined later. QNO addition showed a slight inhibition of activity that may be explained on the basis that it may behave as a weak uncoupler. KCN does not affect any of the activities at all, as expected. The four strains JM83, JM83pDC21F$^-$, W6, and W6pDC21F$^-$ showed a similar pattern of behavior in the presence of the different agents, indicating that this effect is not strain specific. Again the plasmid containing strains have higher specific activity than the non-plasmid containing strains with the former at about 0.06 units/mg protein and the latter at 0.02 units/mg protein. A representative set of data is shown from the nine times these assays were performed.

3. Energization of aerobic-dependent transhydrogenase activity

Transhydrogenase can also be energized by the respiratory chain as seen in fig. 6 (74). A study of this process provided very different results from those seen in the ATP-dependent transhydrogenase assays.

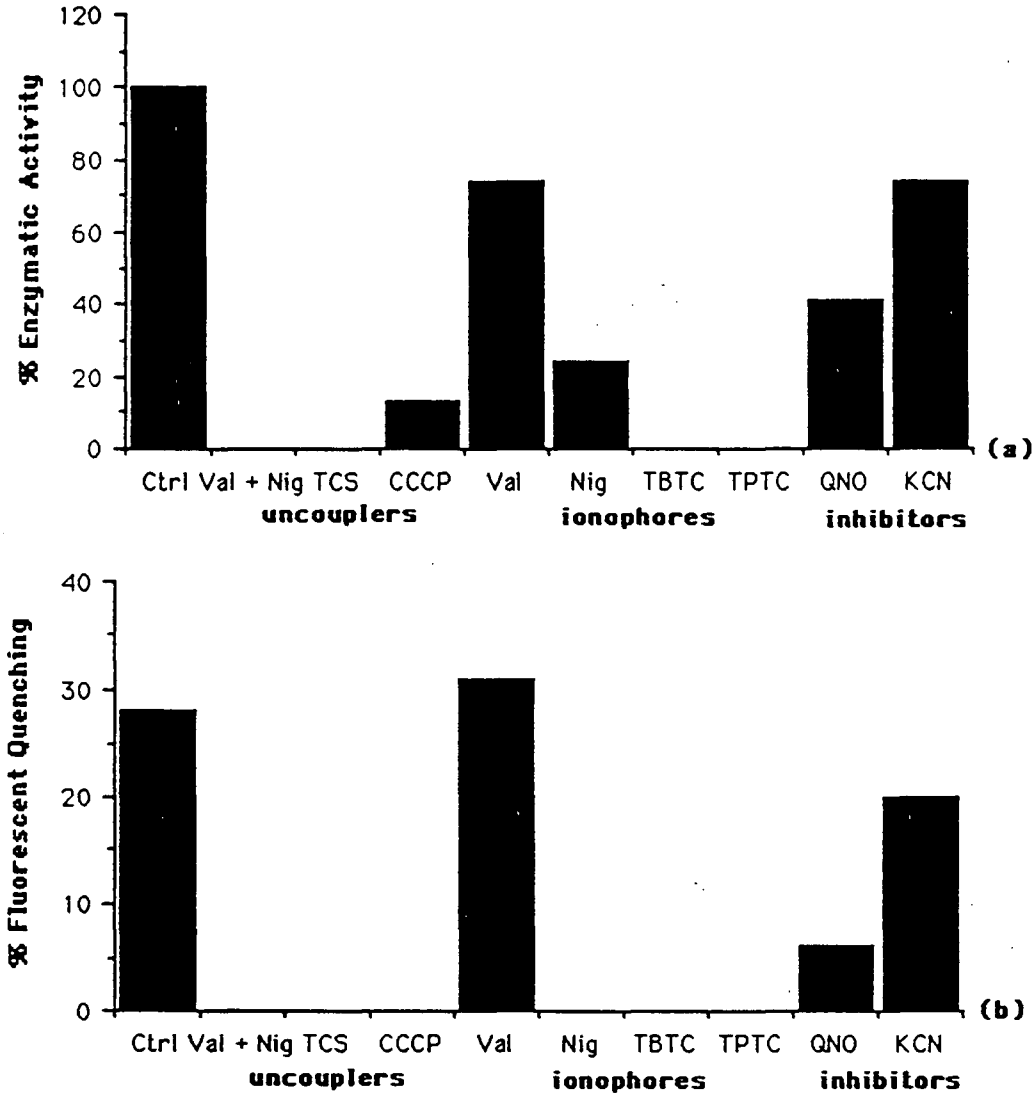


Fig. 9: Effect of uncouplers, ionophores, and respiratory chain inhibitors on ATP-dependent transhydrogenase activity and ATP-dependent proton translocation in inverted membrane vesicles of strain JM83pDC21

Measurement of the ATP-dependent transhydrogenase activity (a) and ATP-dependent proton translocation by fluorescence quenching (b) are as described in Materials and Methods. 320 µg and 3.2 mg of protein was added to each assay mixture for (a) and (b) respectively. The agents of val+nig, TCS, CCCP, val, nig, TBTC, TPTC, QNO and KCN were added independently to the final concentrations of 4.5+7, 2, 10, 9, 14, 25, 25, 100 µM and 10 mM, respectively. The control (100%) value in (a) was 0.058 units/mg protein.

Fig. 10(a) shows the aerobic-dependent activities of the plasmid containing strain JM83pDC21F$^-$ measured in the presence of different agents. Similar results were obtained with the strain W6pDC21F$^-$. The control assay is as before. Uncouplers stimulated the aerobic-dependent activities. This is in stark contrast to the ATP-dependent activities and, as will be shown later, the aerobic-dependent activity of non-plasmid containing strains. The stimulation represents a chemiosmotic anomaly. Valinomycin did not have any effect on the enzyme activity similar to the results of the ATP-dependent assays. In this experiment, nigericin stimulated the enzyme activity only slightly but in other sets of data (not shown), nigericin has stimulated the activity several-fold. It has a similar effect as the uncouplers. Both TBTC and TPTC inhibited the activity as in the ATP-dependent assays. Surprisingly, neither QNO or KCN inhibited the aerobic-dependent activities although both are respiratory chain inhibitors. In fact, KCN stimulated the aerobic-dependent activity. Comparable results to those just described were seen for the non-plasmid containing strains of JM83 and W6 in fig. 11(a) with the exception that uncouplers and nigericin inhibited the aerobic dependent activities in these cases.

The fluorescence quenching profiles of fig. 10(b) and 11(b) are essentially similar to that found in fig. 9(b) for ATP-dependent fluorescence quenching. The startling fact is that all three profiles are similar. The chemiosmotic anomaly of uncoupler stimulated aerobic-dependent transhydrogenase activity in the plasmid containing strains does not have corresponding anomalous effects in its proton translocation assays. Protons are dissipated by uncouplers in the aerobic-dependent fluorescence quenching assays of the plasmid containing strains (fig. 10b). Therefore, the aerobic-dependent transhydrogenase activity can be stimulated in the absence of a proton gradient for the plasmid containing strains. These facts provide evidence against Mitchell's theory. All of the above experiments were performed five times and a representative set of data is shown.

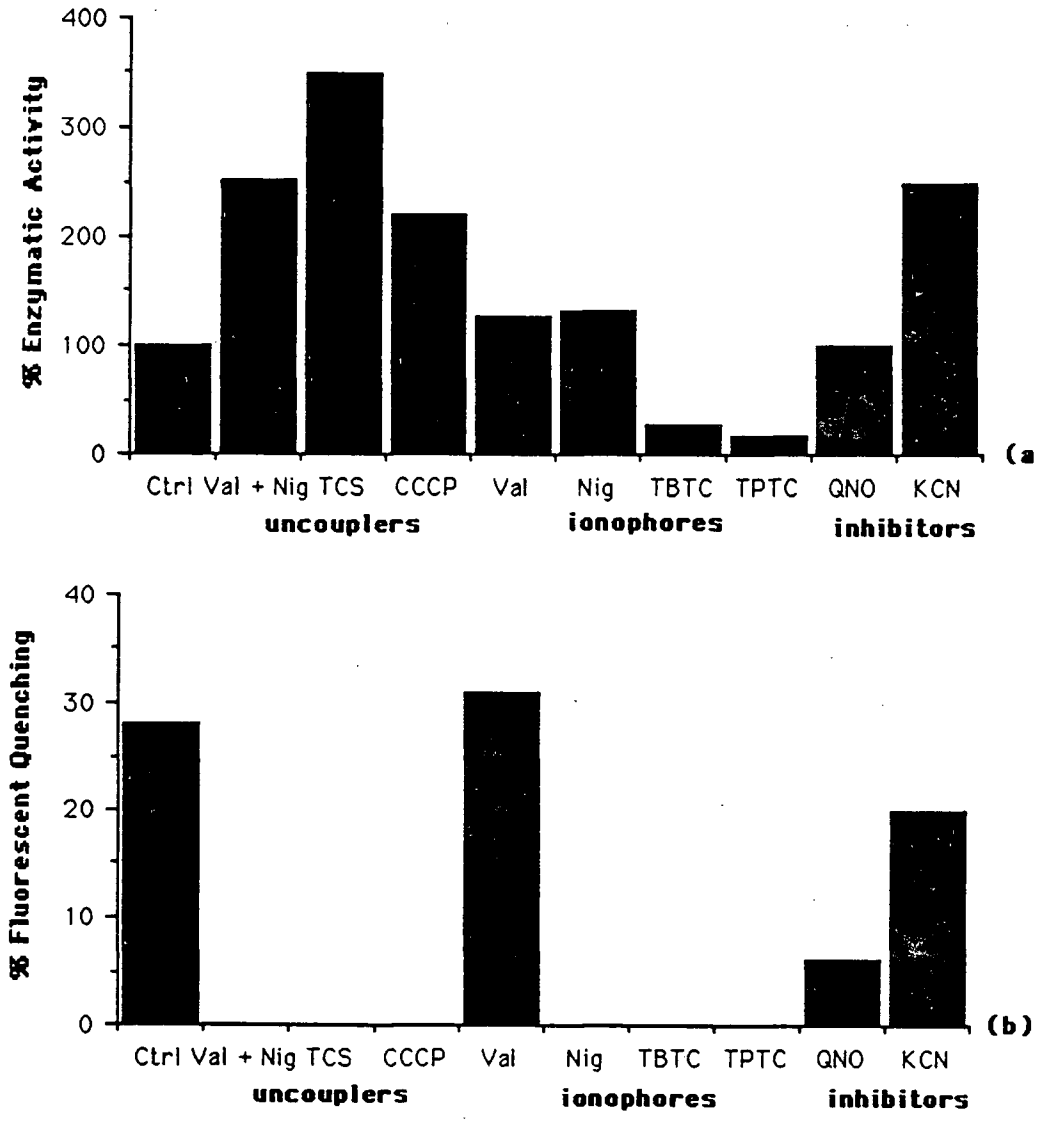


Fig. 10: Effect of uncouplers, ionophores, and respiratory chain inhibitors on aerobic-dependent transhydrogenase activity and respiratory-dependent proton movement in inverted membrane vesicles of the plasmid containing strain JM83pDC21

Measurement of the aerobic-dependent transhydrogenase activity (a) and aerobic-dependent proton translocation by fluorescence quenching (b) are as described in Materials and Methods. 320 µg and 3.2 mg of protein was added to each assay mixture for (a) and (b) respectively. The agents of val+nig, TCS, CCCP, val, nig, TBTC, TPTC, QNO and KCN were added independently to the final concentrations of 4.5+7, 10, 10, 9, 14, 50, 100, 100 µM and 10 mM, respectively. The control (100%) value in (a) was 0.044 units/mg protein.

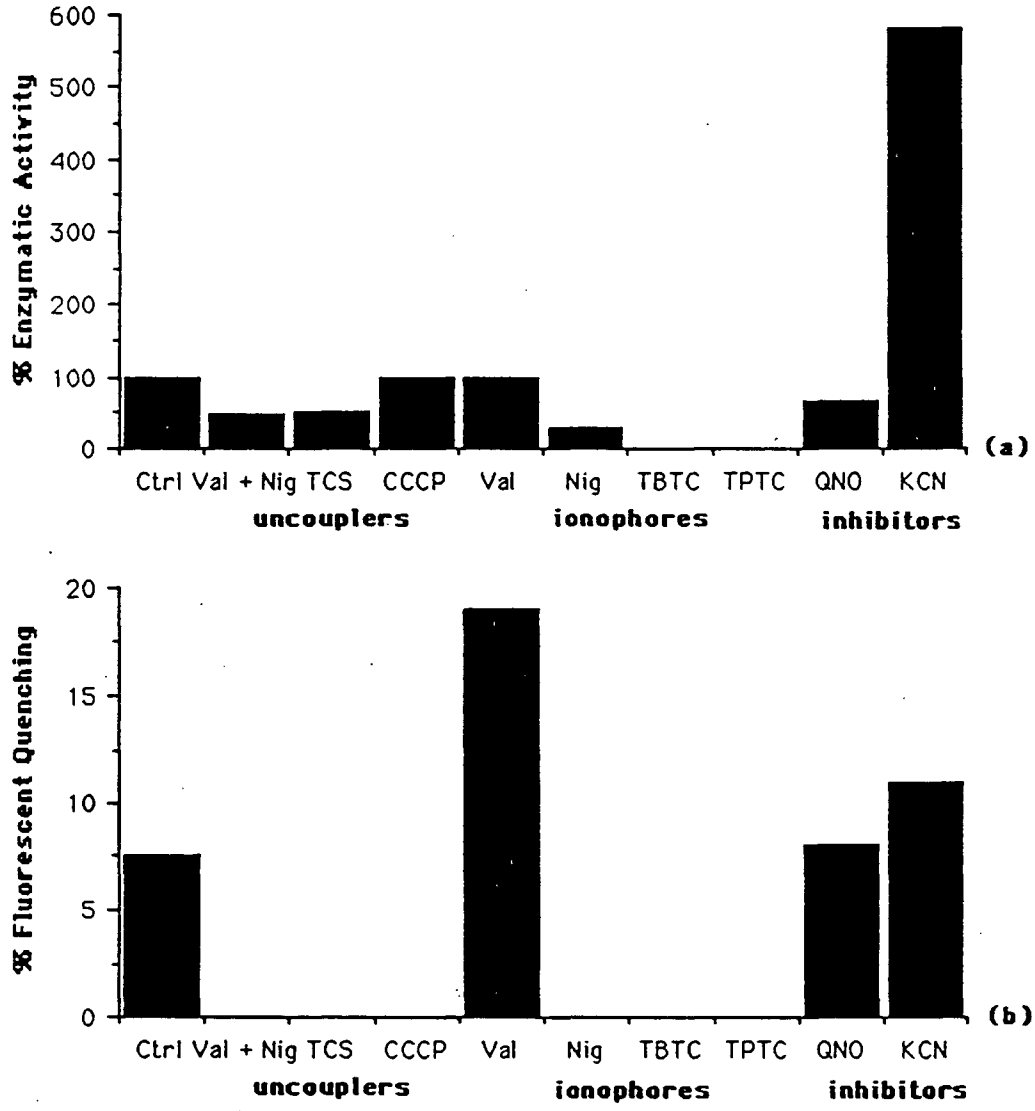


Fig. 11: Effect of uncouplers, ionophores, and respiratory chain inhibitors on aerobic-dependent transhydrogenase activity and respiratory-dependent proton movement in inverted membrane vesicles of non-plasmid containing strain JM83

Measurement of aerobic-dependent transhydrogenase activity (a) and aerobic-dependent proton translocation by fluorescence quenching (b) are as described in Materials and Methods. 240 µg and 2.4 mg of protein was added to each assay mixture for (a) and (b) respectively. The agents of val+nig, TCS, CCCP, val, nig, TBTC, TPTC, QNO and KCN were added independently to the final concentrations of 4.5+7, 10, 10, 9, 14, 50, 100, 100 µM and 10 mM, respectivley. The control (100%) value in (a) was 0.014 units/mg protein.

C. The components and activities of the respiratory chain from plasmid and non-plasmid containing strains of *E. coli*

1. NADH (d-NADH) oxidase activity and inhibitor effects on the respiratory chain

While assaying the aerobic dependent activities of the plasmid strains, it was discovered that these strains did not have the characteristic NADH oxidase phase at the beginning of the enzyme activity curves (fig. 7). Since these strains showed the chemiosmotic anomaly described above, their oxidase activity was examined. Table I shows the NADH oxidase activity of inverted vesicles from strains JM83 and JM83pDC21F$^-$ measured from the time to deplete dissolved oxygen in the assay medium. The activity is approximately 3-times greater in the non-plasmid strain than in the plasmid containing strain, confirming our original suspicion. Because the bacterial respiratory chain can also oxidize d-NADH, possibly through a slightly different pathway, the d-NADH oxidase activity was also measured. Table I indicates that there was little difference between the d-NADH oxidase activities of the non-plasmid and the plasmid containing strains. The same activities were remeasured in Table II using a different methodology that involved measuring the rate of NADH oxidation spectrophotometrically from the change in absorbance at 340 nm. NADH oxidase activity was 5-times higher in the non-plasmid strains than the strains with the plasmid. d-NADH oxidase activity could not be determined.

The surprising discovery from the previous section that neither QNO nor KCN inhibited aerobic-dependent activity prompted an investigation to assess the effectiveness of these agents as respiratory chain inhibitors. Table I presents the data concerning the effect of these inhibitors on NADH oxidase activity. These inhibitors, at the concentration used in the transhydrogenase assays, were indeed effective in inhibiting the respiratory chain activity for both the plasmid and non-plasmid strains. Therefore, the ineffectiveness of the inhibitors on the aerobic-

Table I: Oxidase activity with various substrates of the respiratory chain

Strains	Oxidase Activity (μg atoms O/min/mg protein)				
	NADH	d-NADH	NADH + QNO	NADH + KCN	PMS/Asc
JM83	0.143	0.0086	0.0319	0.0614	0.086
JM83pDC21	0.055	0.0061	0.0073	0.0486	0.073

The concentration of substrates added and the measurement of the enzyme assays are as described in Materials and Methods. QNO and KCN were added to the final concentrations of 100 μM and 10 mM where indicated. Oxidase activity was calculated from the time taken to deplete oxygen in the assay medium.

Table II: Reductase and oxidase activity of the bacterial NADH dehydrogenase complex

Strains	Enzyme Activities (units/mg protein)					
	Ferricyanide reductase		Q_1 reductase		NADH oxidase	
	NADH	d-NADH	NADH	d-NADH	NADH	d-NADH
JM83	0.931	0.198	0.385	0	0.272	0
JM83pDC21	0.930	0.193	0.381	0	0.051	0

The concentration of the substrates added and the measurement of the enzyme assays are as described in Materials and Methods. NADH oxidase and d-NADH oxidase activity were measured spectrophotometrically from the decrease in absorption at 340 nm.

Table III: Quinone content of inverted bacterial membrane vesicles

Strains	nmole Q/mg protein
JM83	2.3
JM83pDC21	2.5

Quinone was extracted from inverted membrane vesicles and its amount measured as described in Materials and Methods.

dependent activity must reflect the mechanism of energization between the respiratory chain and transhydrogenase or aspects of the assay system itself.

2. Investigating the cause of the low NADH oxidase activity of the plasmid containing strains

Following the discovery of the difference in oxidase activity between the plasmid and non-plasmid containing strains, it was decided that the separate components of the respiratory chain should be investigated in isolation in attempt to pinpoint the cause.

The NADH dehydrogenase activity was the first to be examined. There are two separate NADH dehydrogenases in the bacterial respiratory chain with very different properties (35). NADH dh I can be assayed for by its ability to reduce ferricyanide whereas NADH dh II reduces ubiquinone-1 (36). Table II shows that NADH dh I oxidized both NADH and d-NADH and there was no difference between the non-plasmid and plasmid containing strain activities. NADH dh II, on the other hand, oxidized only NADH and not d-NADH, but it too showed no difference between the non-plasmid and plasmid strain activities. So it appears that both types of strains have very similar if not identical NADH dehydrogenases.

The next segment of the respiratory chain after the dehydrogenases is the quinones. They were extracted from both strains and compared for their relative amounts as shown in Table III. Both the plasmid and non-plasmid strains contained approximately the same amount of ubiquinones.

The cytochrome level and content of the strains were analyzed by way of dithionite reduced minus hydrogen peroxide oxidized difference spectra (fig. 12 and 13). Fig. 12 shows that both strains contained similar amounts of cytochromes. There appears to be a little more cytochrome d in the plasmid containing strain than in the non-plasmid strain. This is interpreted from the peaks at 628 and 650 nm in

fig. 12(b) which originate from the oxygen reacting heme of cytochrome d. The cytochrome b content was analyzed by expanding the alpha band region of 515-590 nm (fig. 13). The difference spectra of both strains were similar (fig. 13a and b). The fourth order finite difference spectra gave three characteristic peaks at 556, 558, and 565 nm (fig. 13c and d). The peak at 556 nm is probably due to a mixture of b type cytochromes from succinate dehydrogenase and cytochrome o complex. The peak at 558 nm could possibly be due to cytochrome o with a small mixture of the cytochrome d complex. Finally, the peak at 565 nm is due to cytochrome o (29, 30). These characteristic peaks are similar for both the plasmid and non-plasmid containing strains indicating that the cytochrome content does not differ greatly in the two.

After demonstrating that there is little difference between the respiratory components of the plasmid and non-plasmid strains, the PMS/ascorbate oxidase activity was measured. PMS/ascorbate can reduce the cytochromes directly, bypassing the NADH dehydrogenases and quinones. Its oxidase activity is shown in Table I. The PMS/ascorbate oxidase activity was similar in both strains. Therefore, the low NADH oxidase activity of the plasmid strains may be due to the part of the respiratory chain before the cytochromes.

3. Investigating the presence of a sodium respiratory chain

It has been shown that under certain circumstances, sodium ions can substitute for protons as the coupling ion (103). The presence of a Na^+-coupled oxidative phosphorylation system in the plasmid containing strains could provide an explanation for some of the anomalies observed. This possibility was investigated by placing the inside-out membrane vesicles of the plasmid containing strains in the presence of an external 150 mM NaCl gradient and measuring the energy-independent, energy-dependent transhydrogenase activities and the NADH

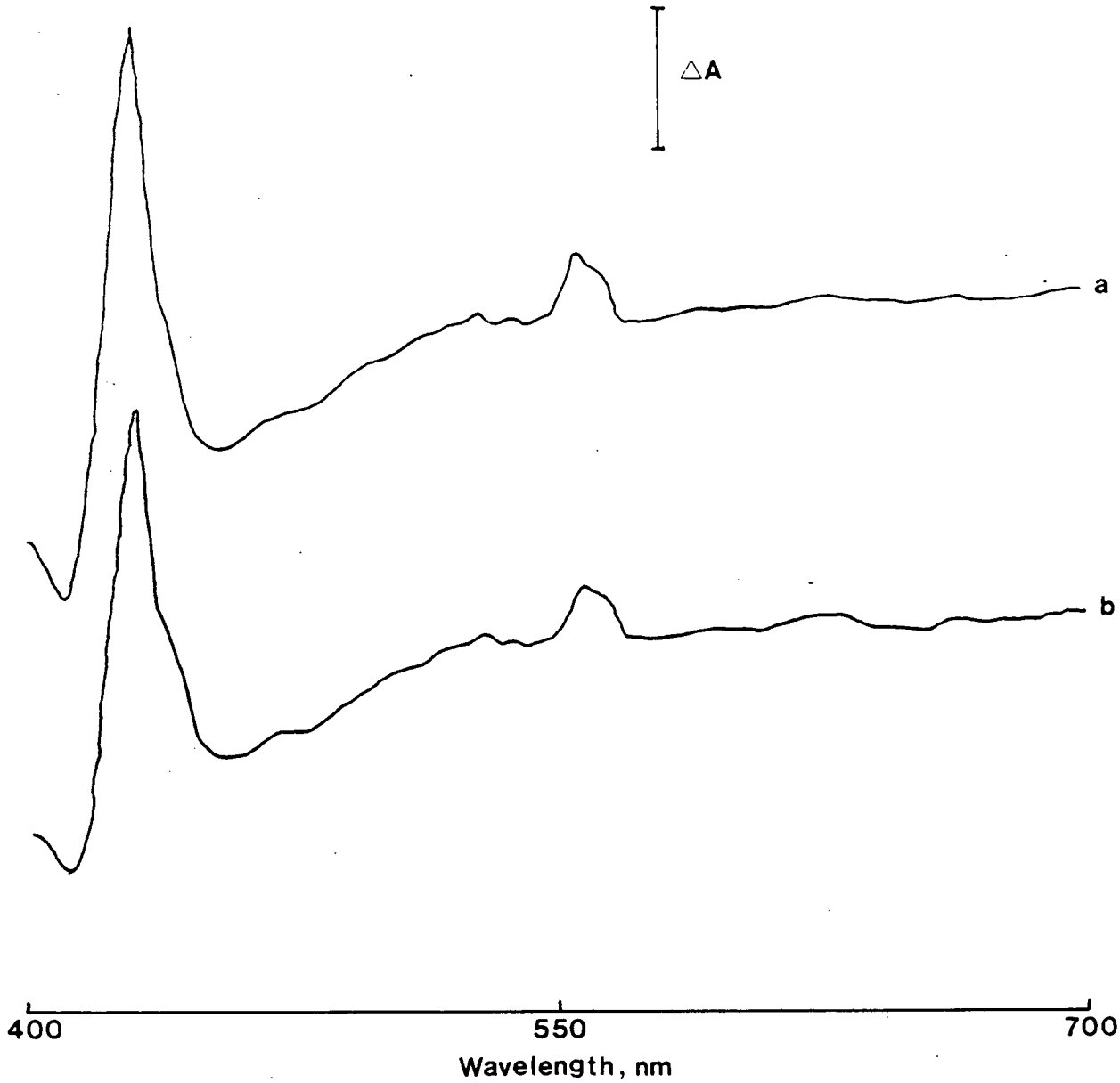


Fig. 12: Comparison of the cytochrome content of the non-plasmid strain JM83 to the plasmid containing strains of JM83pDC21

'Dithionite reduced minus H_2O_2oxidized' spectra of the cytochrome content in non-plasmid and plasmid strains is compared at 77 K. Curve a is representative of the non-plasmid strains of JM83 and W6 whereas curve b is representative of the plasmid containing strains of JM83pDC21F$^-$ and W6pDC21F$^-$. The bar represents an absorbance of 0.1. The membrane concentrations were 14.5 and 14.3 mg protein/ml for curve a and b respectively.

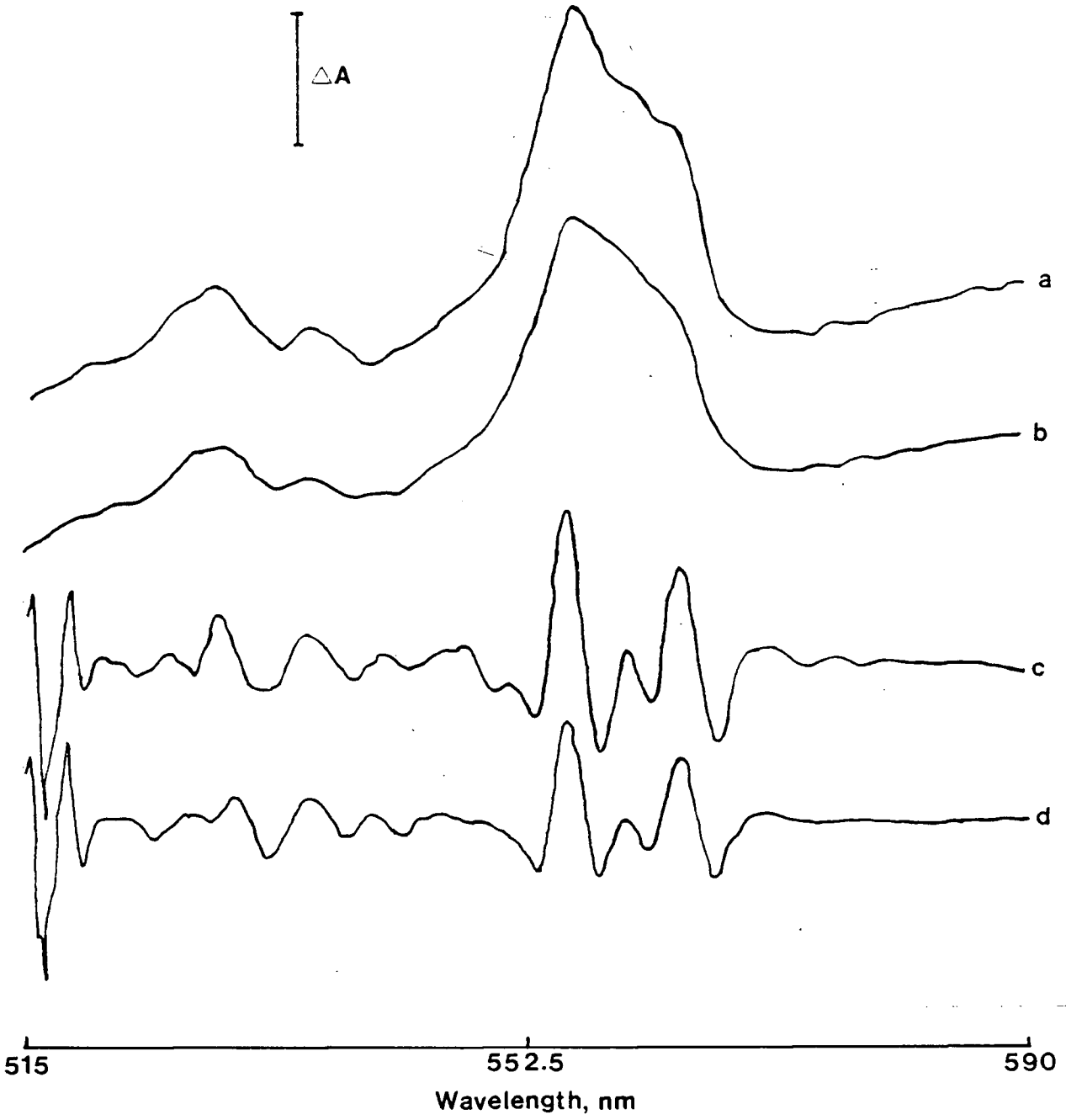


Fig. 13: Detailed analysis of the cytochrome alpha band region in non-plasmid and plasmid containing strains JM83 and JM83pDC21

Curves a and b correspond to the 'dithionite reduced minus H_2O_2 oxidized' difference spectra of non-plasmid and plasmid strains respectively. Curves c and d are fourth-order finite difference spectra derived from the samples giving curves a and b. The bar represents an absorbance of 0.02. The membrane protein concentrations were 14.5 mg protein /ml (curves a and c) and 14.5 mg protein/ml (curves b and d).

oxidase activity (data not shown). No significant change in any of the activities was observed upon imposition of the NaCl gradient. Addition of the Na^+/H^+ antiporter, monensin, caused effects on the enzyme that are readily explainable by proton-motive force but not by a sodium-motive force. Identical experiments were done on the non-plasmid containing strains as a control. They showed similar results. Thus, it is concluded that the chemiosmotic anomaly is not caused by the presence of a sodium respiratory chain.

D. The high transhydrogenase content of plasmid containing strains

1. Verifying the high transhydrogenase content of the plasmid containing strains

Since the aerobic dependent anomaly does not appear to be due to the respiratory chain, it may be caused by overcrowding the membrane with transhydrogenase enzymes. The constituent proteins of the membrane vesicles of the four strains JM83, JM83pDC21F$^-$, W6, and W6pDC21F$^-$ were electrophoretically sieved on a SDS-PAGE gel. Fig. 14 shows that the plasmid containing strains expressed a significantly higher level of the bacterial transhydrogenase dimer (49 and 54 kdal) than the non-plasmid strains. This result is supported by the western blot run for the same strains (fig. 15). Therefore, there is no doubt that the plasmid strains do express a higher level of transhydrogenase in the membrane.

If the high level of transhydrogenase is the cause of the aerobic-dependent anomaly, one should be able to demonstrate a gradient of response to this effect by proportionally increasing the lipid to protein ratio. This was attempted by sonicating *E. coli* membrane lipids with JM83pDC21F$^-$ membrane vesicles. No gradient of response was found. These results, however, are inconclusive because it could not be determined if the membranes did fuse together to form new vesicles with higher lipid : protein ratio.

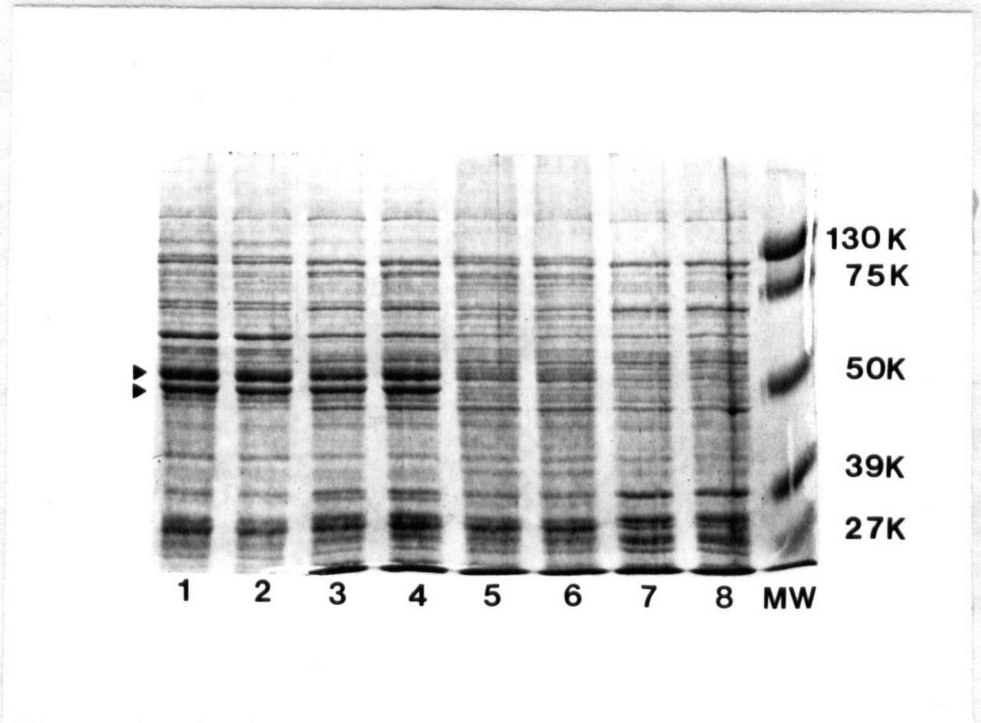


Fig. 14: SDS polyacrlamide gel electrophoresis of membrane proteins from the plasmid and non-plasmid containing strains

10 μg of membrane proteins were added to an equal volume of SDS sample buffer and loaded onto each lane without heating. Samples were run on a 5%/10% discontinuous SDS polyacrylamide gel and proteins stained with Coomassie-blue. Lanes 1 and 2, W6pDC21F$^-$; lanes 3 and 4, JM83pDC21F$^-$; lanes 5 and 6, W6; lanes 7 and 8, JM83. MW stands for the molecular weight markers. For further details refer to the Materials and Methods section. The α and β subunits of the transhydrogenase enzyme are indicated by arrow heads.

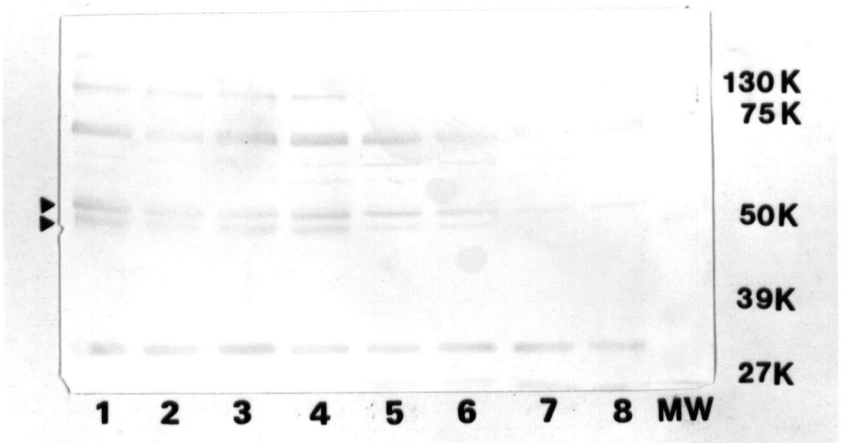


Fig. 15: Western blot of membrane proteins from the plasmid and non-plasmid containing strains

A SDS polyacrylamide gel identical to that in fig.10 was run and the proteins electrophoretically transferred to nitrocellulose. The blot was reacted to polyclonal antibodies to the transhydrogenase dimer and the position of the bound antibodies recogized as described in Materials and Methods. Lane 1 and 2, W6pDC21F^{-}; lane 3 and 4, JM83pDC21F^{-}; lane 5 and 6, W6; lane 7 and 8, JM83. MW stands for the molecular weight standards. For further details refer to the Materials and Methods.

Lastly, it was of interest whether the property of low oxidase activity and uncoupler stimulated aerobic dependent activity could be conferred to a strain by transforming the strain with the transhydrogenase gene containing plasmid (pDC21). This was performed on the W6 strain to create the W6pDC21F- strain (data not shown). As the results from above have demonstrated, one can indeed transfer these properties by transforming the strain (Note: All the data previously shown for the W6pDC21F- strains have come from this transformation). Thus, it is likely that the unique properties aforementioned are a direct consequence of having the plasmid in the cell.

2. Separation of membrane vesicles containing high levels of transhydrogenase

Weiner et al. (75) found that when they amplified the fumarate reductase enzyme in *E. coli* by 30-fold, greater than 50% of the inner membrane protein was fumarate reductase. Furthermore, the cell accomodated this extra protein by inducing intracellular tubular membrane structures. Since a similar situation exists in my system where the transhydrogenase enzyme is amplified by 70-fold, one might expect tubular structures also to exist in the cells of the plasmid containing strains. This was confirmed by Clarke (72). It may be possible that the chemiosmotic anomaly of the plasmid strains is due to a very different form of energization which exists in these tubular structures. Thus, it was decided to separate the membrane vesicles via a shallow sucrose gradient. This had been successfully done previously by Young and Jacoby (76) for *E. coli* membrane vesicles.

Fig. 16 shows a SDS-PAGE gel of the fractions from the sucrose gradient. It is easily observable that separation had been achieved. Fig. 17 reveals that the majority of the transhydrogenase containing vesicles were in fractions 1 and 2 and the majority of the respiratory chain containing vesicles were in fractions 6-8. Ferricyanide, which is an electron acceptor for NADH dh I, was used as a marker for

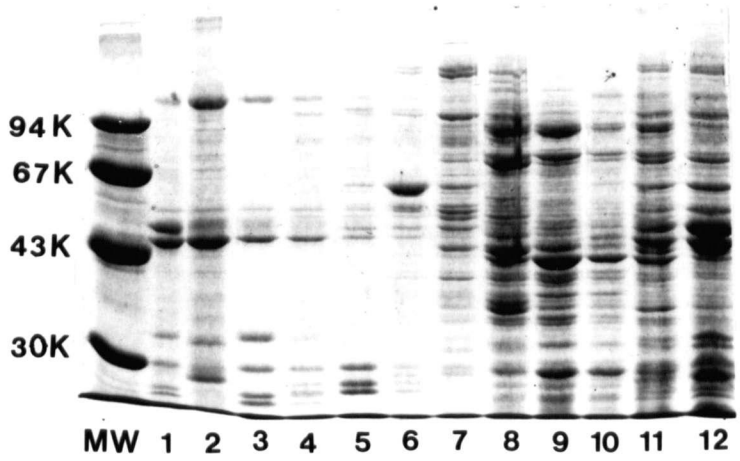


Fig. 16: Separation of *E. coli* membrane vesicles by sucrose density centrifugation

JM83pDC21F$^-$ membrane vesicles were fractionated on a 20-45% linear sucrose gradient and collected in 0.5 ml fractions. 7.5 µl of each fraction mixed with an equal volume of SDS sample buffer was loaded onto lanes 1-10 of the gel corresponding to the most dense fractions to the least dense. Lanes 11 and 12 were run as controls of the French pressed and ultracentrifuged membrane vesicles respectively. MW stands for the molecular weight markers. Samples were electrophoresed on a 5%/10% discontinuous SDS polyacrylamide gel and stained with Coomassie-blue. A similar pattern of separation is seen for the JM83 membrane vesicles (data not shown). For further details refer to the Materials and Methods section.

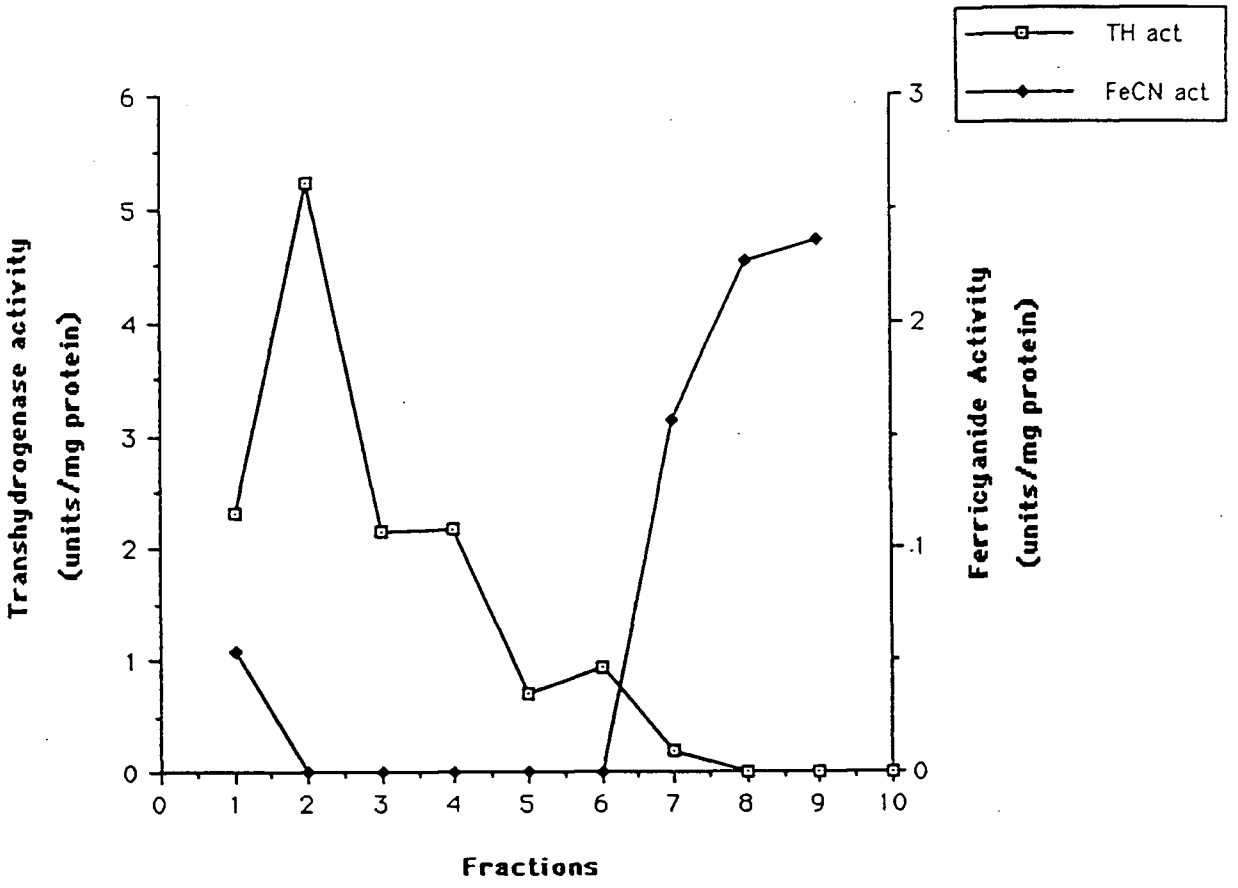


Fig. 17: Transhydrogenase and NADH-ferricyanide reductase activities of sucrose density gradient fractions

Energy independent transhydrogenase and NADH-ferricyanide reductase activities were measured for the fractions as described by Materials and Methods. The activities of fractions 1-10 correspond to lanes 1-10 in the SDS polyacrylamide gel shown in Fig. 13.

the respiratory chain. Due to the low yield and dilute concentration of these fractions, very few other assays could be performed. It should be noted that the membrane vesicles of the non-plasmid containing strains of JM83 separated identically to its plasmid containing strains (fig. 16 and 17). The significance of these results shall be discussed later.

Lastly, it was found that plasmid containing strains have a lower growth rate (fig. 18) and yield of cells than the non-plasmid containing strains. von Meyenburg et al. (77) had also reported abnormal physiological and morphological effects in *E. coli* that overproduce ATP synthase by 10 to 12-fold. They found these cells to have pronounced inhibition of cell division and growth. The reason for these effects is unclear.

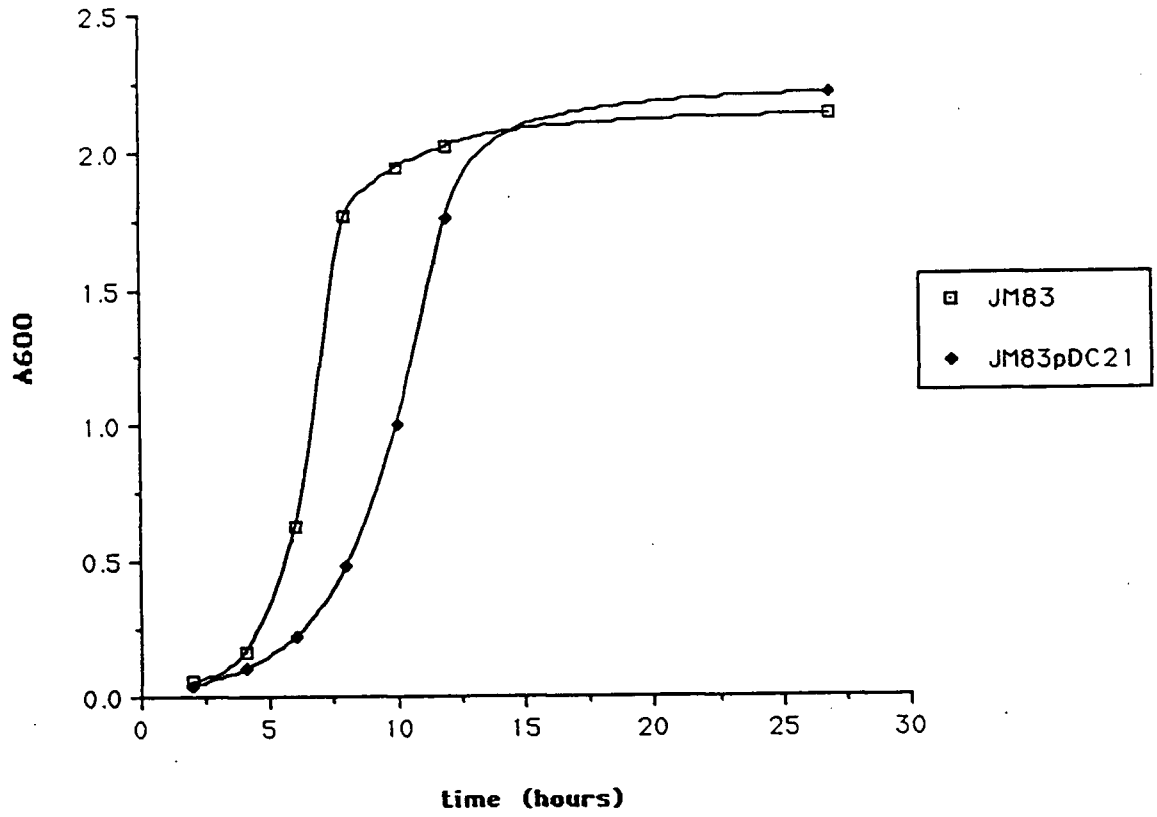


Fig. 18: Growth curves of plasmid and non-plasmid containing strains

The bacterial strain JM83 and JM83pDC21F⁻ were grown overnight as starter cultures in 10 ml PAB tubes. They were then added as a 2% innoculum into M9 medium and grown at 37 °C with shaking (250 rpm). Growth was measured as the absorbance of the culture at 600 nm. A similar pattern is seen for the curves of W6 and W6pDC21F⁻ (curves not shown).

IV DISCUSSION

A. Energization of transhydrogenase

Mitchell and Moyle (8) demonstrated that the reduction of NAD^+ by NADPH is coupled to an uptake of protons in bovine-heart submitochondrial particles. The question posed here is whether transhydrogenation is primarily controlled by the proton gradient (ΔpH), the membrane potential ($\Delta\psi$), or a combination of both. Kaback and co-workers (78) have shown that, in *E. coli*, some active proton-symport systems are driven exclusively by proton gradients whereas others respond to $\Delta\psi$. Rydstrom (46) has reported a transient phase of high initial NADPH to AcNAD activity in transhydrogenase proteoliposomes with an internal pH of 8 and an external pH of 6. His experiment implicated the importance of a transmembrane pH gradient in transhydrogenation. Other studies by Earle and Fisher (47) provided evidence that the membrane potential is also important. They loaded proteoliposomes with K^+ or Na^+ and assayed activity of transhydrogenase in a medium containing Na^+ or K^+ respectively. Addition of valinomycin gave high rates of NADPH to NAD^+ and NADH to $NADP^+$ transhydrogenation when K^+ movement occured in a direction opposite to that proposed for transhydrogenase-coupled proton movements. Furthermore, they claimed that pH gradients had very little effect in transhydrogenation.

In contrast to the above experiments which were done with artificial proteoliposomes and purified transhydrogenase, the work done in this thesis has concerned the energetics of everted, native membrane vesicles. Fig. 8(a) showed that uncouplers stimulated transhydrogenase activity suggesting that respiratory control may exist in these vesicles from a balancing of the electrochemical hydrogen ion gradient across the membrane against the substrate oxidation-reduction potential. Dissipating the proton gradient, as evidenced by the relieving of the fluorescence quenching with uncouplers (fig. 8b), removes this control and consequently transhydrogenation is stimulated. These data establish that a electrochemical

gradient is involved in transhydrogenation but it does not reveal whether ΔpH or $\Delta\psi$ or both are important in this process. The importance of ΔpH and $\Delta\psi$ is unveiled by the results of experiments with the ionophores valinomycin and nigericin (added separately). These results imply that ΔpH is the most important component of the electrochemical gradient which drives transhydrogenase and not $\Delta\psi$.

TBTC and TPTC's effects on the energy independent assays do not agree with the above explanation. This may be rationalized by arguing that these agents do not function as OH^-/Cl^- exchangers in the assays as previously mentioned. Singh and Bragg (69) have proposed an alternative mechanism of action for TBTC on transhydrogenase energetics. They suggest that TBTC may be a sulfydryl reacting agent that acts directly on the transhydrogenase. Since critical sulfydryl groups exist on the transhydrogenase (79), TBTC's action may well inhibit both the enzymatic and proton-translocating ability of the enzyme. TPTC should have a similar effect because it differs from TBTC only in having phenyl groups instead of butyl groups in its structure making it more membrane soluble than its counterpart.

B. Energization of ATP-dependent activity

ATP dependent transhydrogenation was found to be inhibited by uncoupler (80) and specifically by inhibitors of phosphorylation such as oligomycin and DCCD (81). Because of these properties, ATP was hypothesized to drive the transhydrogenase reaction by creating a $\Delta\mu_H$ as predicted by Mitchell. However, in submitochondrial particles, Ernster et al. (82) have demonstrated a direct relationship between inhibition of the ATPase activity with increasing amounts of the Pullman-Monroy inhibitor and inhibition of the ATP-linked transhydrogenase reaction. This relationship exists in spite of the large excess of ATPase over transhydrogenase. The classical chemiosmotic theory would not have predicted this because inhibiting the excess ATPase should not adversely affect the $\Delta\mu_H$ which energizes the ATP-

dependent transhydrogenation. These findings were interpreted as a localized interaction between ATPase and transhydrogenase with each ATPase being able to "energize" transhydrogenase in a limited domain within the membrane. Furthermore, Kay and Bragg (83) have isolated a mutant strain of *Salmonella typhymurium* HfrA that cannot perform ATP-dependent transhydrogenation but does have a functional transhydrogenase and is capable of carrying out all its other energy-dependent processes including ATP-dependent quenching of atebrin fluorescence. The properties of this mutant are also difficult to explain in a chemiosmotic manner. Therefore, some controversy exists about the mechanism of ATP-dependent transhydrogenation.

The work in this thesis investigated the ATP-dependent energization of everted, *E. coli* membrane vesicles. Fig. 9 indicated that the addition of uncouplers inhibited this reaction and dissipated fluorescence quenching for both the plasmid and non-plasmid containing strains to a similar extent. These results argue for a bulk proton gradient to be the energy intermediate between ATPase and transhydrogenase. Furthermore, the effects of valinomycin and nigericin (added separately) suggest once more that ΔpH is the important component of the electrochemical gradient in transhydrogenase.

The ability of both TBTC and TPTC to inhibit both the enzymatic and fluorescence quenching activities support the previous notion that these compounds are sulfydryl agents which act directly on the transhydrogenase. If they behaved as electroneutral OH^-/Cl^- exchangers, they would have stimulated the ATP-dependent reaction and maintained or stimulated fluorescence quenching by increasing the concentration of protons inside the membrane vesicle.

Lastly, inhibitors of electron transport were found not to significantly affect the ATP-driven transhydrogenase reaction in agreement with Ernster and Danielson's work (84) with submitochondrial particles.

C. Energization of aerobic-dependent activity

Comparatively little work has been done on the aerobic-driven transhydrogenase reaction. The reason is that no simple system has yet been developed to study this process in a systematic way. The work described in this thesis implicates the existence of a localized pathway between the respiratory chain components and transhydrogenase in *E. coli* membrane vesicles.

Fig. 10 and 11 show that addition of uncouplers stimulated the aerobic-dependent activities of transhydrogenase in the plasmid containing strains but inhibited the same activities in the non-plasmid containing strains. The chemiosmotic theory would have predicted an inhibition of the enzymatic activities for both cases since the uncouplers should dissipate the aerobically generated internal protons used to energize the transhydrogenase reaction (see fig. 6). One explanation may be that the uncouplers do not fully dissipate the proton gradients in the plasmid strains. Although the fluorescent quenching data (fig. 10) show that indeed the proton gradient is relieved upon uncoupler addition, nevertheless, one must be careful with the interpretation of these data. For example, fluorescent probes may not probe the internal pH of the vesicle exclusively, but may also interact with the surface or the interior of the membrane (85). Quinacrine has been proposed to act by decreasing its fluorescence in response to binding to the "energized" membrane via its diprotonated form (86). The other explanation is that a localized interaction exists between the respiratory components and transhydrogenase in the plasmid containing strain due to higher levels of transhydrogenase protein. This interaction may not exist or may occur to only a very limited extent in the non-plasmid containing strains.

The results of experiments with the ionophores agree with the interpretation that the ineffectiveness of valinomycin indicates that $\Delta\psi$ is not significant in this process. The observation that the presence of nigericin did not affect the aerobic-dependent transhydrogenase activity of the plasmid containing strains but inhibited

the activity of the non-plasmid containing strains, confirms the involvement of ΔpH in this "localized interaction". The inability of nigericin to dramatically stimulate the enzymatic activity as did the uncouplers (fig. 10a) may be due to a difference in mechanism between these two classes of compounds. The effects of TBTC and TPTC can probably be explained as for the case of ATP-dependent activities.

An interesting fact is that neither KCN nor QNO inhibited the aerobic dependent activities of both the plasmid and non-plasmid containing strains (fig. 10 and 11) even though the concentrations used were effective in blocking the NADH oxidase activity (table I). This indicates that the aerobic dependent energization process does not require a fully functional respiratory chain.

Another anomaly is that the plasmid strains contain a much lower NADH oxidase activity than do the non-plasmid strains (table I and II). This effect is not unique. Weiner et al. (75) have also found that cells containing amplified levels of fumarate reductase have a complete absence of NADH oxidase activity in the cell envelope. The low oxidase activities may originate from a segment of the respiratory chain before the cytochromes, as hinted at by the results with PMS/ascorbate (table I), but no direct evidence was obtained to support this.

These anomalous effects together with the great flexibility of *E. coli* to induce different respiratory chains prompted us to inquire whether the effects were due to the induction of a different respiratory pathway in the plasmid strains. The results from tables II and III and figures 12 and 13, indicated that there were no differences between the NADH dehydrogenases, quinone, or cytochrome content of the plasmid strains and the non-plasmid strains. Furthermore, the anomaly could not be explained by the presence of a sodium-translocating respiratory chain.

It appears then that both the plasmid and non-plasmid strains have the same respiratory chain. This is not too surprising considering that different respiratory chains are usually induced only under different growth conditions including the

presence of different terminal electron acceptors and carbon sources. Overcrowding the membrane with transhydrogenase probably does not pose a sufficient challenge to the proper respiratory function of the cell to warrant the induction of a different respiratory chain.

The other explanation of the chemiosmotic anomaly is the presence of the high transhydrogenase content in the membranes of plasmid containing strains as demonstrated by the SDS-PAGE gel, Western blot, and transformation data (fig.14 and 15). It may be that energy transfer occurs by collision between complexes as suggested by Slater and colleagues (26) in their "collision hypothesis". High transhydrogenase levels in the plasmid strain would promote this localized transport because its presence would increase the frequency of collisions. Uncouplers might act in some yet undefined manner in assisting this mode of energization thus stimulating the aerobic dependent activities. Uncouplers probably do not influence the respiratory chain components directly as allosteric compounds because of the wide variation in structure and mechanism of uncouplers that show this chemiosmotic anomaly. Respiratory chain inhibitors are ineffective in this model due to the ability of the individual high energy respiratory components to directly act with transhydrogenase in energy transduction. Furthermore, NADH oxidase activity would be impaired in the plasmid strains because redox pairs would collide less frequently with each other in the presence of the high transhydrogenase content. This model, however, does not exclude the possibility of a bulk proton gradient in energization. It merely hypothesizes this gradient to be a secondary effect and the collision of energized complexes to be the primary driving force (see introduction). In all, a collision model would provide an explanation, albeit unproven at this stage, to a great number of the observations described in this thesis.

D. Heterogeneity among membrane vesicles of *E. coli*

Fig. 16 revealed that membrane proteins are associated with different subsets of membrane vesicles that can be separated by a shallow sucrose gradient. Fig. 17 showed that the transhydrogenase enzymes tend to be localized to one type of membrane vesicle. NADH dh I, and possibly also the other respiratory chain components, tend to gather together in another type. The existence of these associations among vesicles and proteins suggest that there is a situational organization of at least some proteins within the inner membrane of *E. coli* , possibly by segregating proteins into distinct membrane regions or domains. In other words, proteins do not flow freely in the membrane surface as predicted by the fluid mosaic model. This concept is feasible because the bacterial cell must somehow perform site-specific events such as cell division, elongation or maintainance of shape by organizing its proteins in localized domains (87).

The aforementioned separation of different membrane vesicle types was similar for both the plasmid and non-plasmid strains. There was no detectable, specific separation of the induced tubular structures in the plasmid containing strains which would be expected if they contained components that help them perform a distinctly different sort of energetics. However, this separation technique may not give sufficiently resolution to show the difference. Young et al. (88) have used other methods to fractionate bacterial membrane vesicles such as electrophoresis through dilute agarose and sizing chromatography through Sephacryl S-1000. These might yield different results to those described here.

V. CONCLUSION

The work from this thesis demonstrated that strains of *E. coli* containing high levels of transhydrogenase have aerobic-dependent transhydrogenase activity that are insensitive to uncouplers. This effect cannot be explained by the classical

Mitchell chemiosmotic hypothesis. It is concluded that some form of localized interaction between respiratory chain components and the transhydrogenase possibly involving collision between energized complexes, as suggested by Slater et al. (26), may account for the observed results.

VI. REFERENCES

1. Warburg, O. and W. Christian (1939), *Biochem. J.* 303, 40-68

2. Lipmann, F. (1941), *Adv. Enzymol.* 1, 99-162

3. Slater, E.C. (1953), *Nature* 172, 975-978

4. Lardy, H.A., D. Johnson and W.C. McMurray (1958), *Arch. Biochem. Biophys.* 78, 587-597

5. Boyer, P.D. (1965), *Oxidases and related redox systems* (King, T.E., H.S. Mason and M. Morrison Ed.) vol 2, 994-1008, Wiley Press, New York

6. Mitchell, P. (1961), *Nature* 191, 144-148

7. Neumann, J. and A.T. Jagendorf (1964), *Arch. Biochem. Biophys.* 107, 109-110

8. Mitchell, P. and J. Moyle (1965), *Nature (London)* 208, 147-151

9. Jagendorf, A.T. and E. Uribe (1966), *Proc. Natl. Acad. Sci. USA* 55, 170-177

10. Racker, E. and W. Stoeckenius (1974), *J. Biol. Chem.* 249, 662-663

11. Thayer, W.C. and P.C. Hinkle (1975), *J. Biol. Chem.* 250, 5336-5342

12. Montal, M., B. Lee and C.P. Chance (1970), *J. Memb. Biol.* 2, 201-234

13. Decker, S.J. and D.R. Lang (1978), *J. Biol. Chem.* 253, 6738-6743

14. Krulvich, T.A. and A.A. Guffanti (1983), *Adv. Microbiol. Physiol.* 24, 173-214

15. Helgerson, S.L., C. Requadt and W. Stoeckenius (1983), *Biochemistry* 22, 5746-5753

16. Terada, H., O. Shima, K. Yoshida, Y. Shinohara (1990), *J. Biol. Chem.* 265, 7837-7842

17. Story, B.T., D.M. Scott, and C.P. Lee (1980), *J. Biol. Chem.* 255, 5224-5229

18. Williams, R.J.P. (1961), *J. Theor. Biol.* 1, 1-13

19. Williams, R.J.P. (1985), *The enzymology of biological membranes* (Martonosi, A.N., ed.) vol 4, 71-110, Plenum Press, New York

20. Kell, D.B., D.J. Clarke and J.G. Morris (1981), *FEMS Microbiol. Letters* 11, 1-11

21. Prats, M., J. Teissie and J.F. Tocanne (1986), *Nature (London)* 322, 756-758

22. Nagel, J.F. and H.S. Morowritz (1978), *Proc. Natl. Acad. Sci. USA* 75, 298-302

23. Westerhoff, H.V., B.A. Melandri, G. Venturoli, G.F. Azzone, D.B. Kell (1984), *Biochim. Biophys. Acta* 768, 257-292

24. Malpress, F.H. (1984), *Biochem. Soc. Trans.* 12, 399-401

25. Skulachev, V.P. (1982), *FEBS letters* 146, 1-4

26. Slater, E.C., J.A. Berden and H.A. Herweijer (1985), *Biochim. Biophys. Acta* 811, 217-231

27. Boyer, P.D. (1984), *H^+-ATP synthase. Structure, function, regulation* (Papa, S., ed.) 329-338, ICSU Press and Adriatica Editrice, Bari

28. Herweijer, M.A., J.A. Berden and E.C. Slater (1986), *Biochim. Biophys. Acta* 849, 276-287

29. Haddock, B.A. and C.W. Jones (1977), *Bacteriol. Rev.* 41, 47-99

30. Bragg, P.D. (1980), *Diversity of bacterial respiratory systems* (In Knowles, C.J., ed.) 115-136, CRC Press, Florida

31. Kroger, A. (1980), *Diversity of bacterial respiratory systems* (In Knowles, C.J., ed.) 1-18, CRC Press, Florida

32. Stouthamer, A.H., J. van't Riet and L.F. Ottman (1980), *Diversity of bacterial respiratory systems* (In Knowles, C.J., ed.), CRC Press Florida

33. Wallace, B.J., I.G. Young (1977), *Biochim. Biophy. Acta* 461, 84-100

34. Owen, P. and H.R. Kaback (1979), *Biochemistry* 18, 1413-1422

35. Matsushita K., T. Ohnishi and H.R. Kaback (1987), *Biochemistry* 26, 7732-7737

36. Hayashi, M., T. Miyoshi, S. Takashina, T. Unemoto (1989), *Biochim. Biophys. Acta* 977, 62-69

37. E. Hackimi, Z., O. Samuel and R. Azerad (1974), *Biochimie* 56, 1239-1247

38. Haddock, B.A. and H.U. Schairer (1973), *Eur. J. Biochem.* 35, 34-35

39. Newton, N.A., G.B. Cox and F. Gibson (1971), *Biochim. Biophys. Acta* 244, 155-166

40. Kita, K. and Y. Anraku (1981), *Biochem. Int.* 2, 105-112

41. Ashcroft, J.R. and B.A. Haddock (1975), *Biochem. J.* 148, 349-352

42. Poole, R.K. and B.A. Haddock (1975), *Biochem. J.* 152, 537-546

43. Downie, J.A. and G.B. Cox (1978), *J. Bacteriol.* 133, 477-484

44. Rydstrom, J. (1977), *Biochim. Biophys. Acta* 463, 155-184

45. Fisher, R.R. and S.R. Earle (1982), *The pyridine nucleotide coenzymes*, 279-324

46. Rydstrom, J. (1979), *J. Biol. Chem.* 254, 8611-8619

47. Earle, S.R. and R.R. Fisher (1980), *J. Biol. Chem.* 255, 827-830

48. Danielson, L. and L. Ernster (1963), *Energy-Linked Functions of Mitochondria* (Chance B. ed.) 157-175, Academic Press, New York

49. Rydstrom, J., A. Teixeira da Cruz and L. Ernster (1970), *Eur. J. Biochem.* 17, 56-62

50. Yamaguchi, M., Y. Hatefi, K. Trach and J.A. Hock (1988), *J. Biol. Chem.* 263, 2761-2767

51. Clarke, D.M., T.W. Loo, S. Gillam and P.D. Bragg (1986), *Eur. J. Biochem.* 158, 647-653

52. Wakabayashi, S. and Y. Hatefi (1987), *Biochem. Int.* 15, 915-924

53. O' Neal, S.G. and R.R. Fischer (1977), *J. Biol. Chem.* 252, 4552-4556

54. Hatefi, Y., D.C. Phelps and Y.M. Galante (1980), *J. Biol. Chem.* 255, 9526-9529

55. Nicholls, D.G. (1982), *Bioenergetics: An introduction to chemiosmotic theory* (United States ed.) 1-22, Academic Press, London

56. Clarke, D.M. and P.D. Bragg (1985), *J. Bacteriol.* 162, 367-373

57. Jones, C.W. (1982), *Bacterial Respiration and Photosynthesis* (Nelson ed.) 6-10, Thompson Press, New Delhi

58. Lowry, O.H., N.J. Rosegrough, a.L. Farr and A.J. Randall (1951), *J. Biol. Chem.* 193, 265-275

59. Kaplan, N.O. (1967), *Methods Enzymol.* 10, 317-322

60. Fisher, R.J. and D.R. Sanadai (1971), *Biochim. Biophys. Acta* 245, 34-41

61. Kaback, H.R., T. Ohnishi and K. Matsuchita (1987), *Biochemistry* 26, 7732-7737

62. Kroger, A. (1987), *Methods Enzymol.* L III, 579-591

63. Laemmli, U.K. (1970), *Nature (London)* 227, 680-685

64. Towbin, H., T. Staehelin and J. Gordon (1979), *Proc. Natl. Acad. Sci. USA* 76, 4350-4354

65. Harlow, E. and D. Lane (1988), *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York

66. Maniatis, J., E.F. Fritsch and J. Sambrook (1982), *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York

67. Hanstein, W.G. (1976), *TIBS* Mar., 65-67

68. Pressman, B.C. (1976) *Ann. Rev. Biochem.* 45, 501-529

69. Singh, A.P. and P.D. Bragg (1979), *Can J. Biochem.* 57, 1384-1391

70. Kita, K., K. Konishi and Y. Anraku (1984), *J. Biol. Chem.* 259, 3368-3374

71. Huang, C.S., S.J. Kopacz and C.P. Lee (1977), *Biochim. Biophys. Acta* 459, 241-249

72. Clarke, D.M. (1986), *Pyridine Nucleotide Transhydrogenase* (PhD thesis, University of British Columbia), 77-89

73. Lee, C.P. and L. Ernster (1966), *Biochim. Biophys. Acta* 7, 218-234

74. Danielson, L. and L. Ernster (1963), *Biochem. Biophys. Res. Commun.* 10, 91-96

75. Weiner, J.H., B.D. Lemire, M.L. Elmes, R.D. Bradley and D.G. Scraba (1984), *J. of Bacteriol.* 158, 590-596

76. Jacoby, G.H. and K.D. Young (1990), *Analyt. Biochem.* 184, 48-54

77. von Meyenburg, K., B.B. Jorgensen and B. van Deurs (1984), *EMBO J.* 3, 1791-1797

78. Kaback, H.R. (1977), *Biochem. Membr. Transp.* 76, 598-625

79. Yamaguchi, M. and Y. Hatefi (1989), *Biochemistry* 28, 6050-6056

80. Montal, M., B. Chance, C.P. Lee and A. Azzie (1969), *Biochem. Biophys. Res. Commun.* 34, 104-110

81. Keister, D.L. and N.J. Yike (1967), *Biochemistry* 6, 3847-3857

82. Ernster, L., K. Juntti and K. Asami (1972), *J. Bioenerg.* 4, 149-159

83. Kay, W.W. and P.D. Bragg (1975), *Biochem. J.* 150, 21-29

84. Danielson, L. and L. Ernster (1963), *Biochem. J.* 338, 118-205

85. Fiolet, J.W.T., E.P. Bakker and K. van Dam (1974), *Biochim. Biophys. Acta* 368, 432-445

86. Huang, C.S., S.J. Kopacz and C.P. Lee (1977), *Biochim. Biophys. Acta* 459, 241-249

87. Young, K.D. and G.H. Jacoby (1990), *Analyt. Biochem.* 184, 48-54

88. Young, K.D., M.J. Leidenix, G.H. Jacoby, T.A. Henderson (1989), *J. of Bacteriol.* 171, 5680-5686

89. Simoni, R.D. and P.W. Postma (1975), *Annu. Rev. Biochem.* 44, 523-554

90. Hatefi, Y. (1985), *Annu. Rev. Biochem.* 54, 1015-1067

91. Withers, H.K. and P.D. Bragg (1989), *Biochem. Cell Biol.* 68, 83-90

92. Kita, K., K. Konishi and Y. Anraku (1984), *J. Biol. Chem.* 259, 3368-3374

93. Kita, K. and Y. Anraku (1981), *Biochem. Int.* 2, 105-112

94. Hou, C., M. Potier and P.D. Bragg (1990), *Biochim. Biophy. Acta* (In press)

95. Enander, K. and J. Rydstrom (1982), *J. Biol. Chem.* 256, 14760-14766

96. Wu, L.N.Y., R.M. Pennington, T.D. Everett and R.R. Fisher (1982), *J. Biol. Chem.* 257, 4052-4055

97. Carlenor, E., H.L. Tang and J. Rydstrom (1985), *Analyt. Biochem.* 148, 518-523

98. Persson, B., K. Enander, H.L. Tang and J. Rydstrom (1984), *J. Biol. Chem.* 259, 8626-8632

99. Clarke, D.M. and P.D. Bragg (1985), *Eur. J. Biochem.* 149, 517-523

100. Rydstrom, J., B. Persson and H.L. Tang (1984), *Bioenergetics* (Ernster ed.) chp 7, 207-219, Elsevier Science Publisher, B.V.

101. Pennington, R.M. and R.R. Fisher (1981), *J. Biol. Chem.* 256, 8963-8969

102. O'Neal, S.G., S.R. Earle and R.R. Fisher (1980), *Biochim. Biophy. Acta* 589, 217-230

103. Skulachev, V.P. (1989), *J. Bioenerg. Biomemb.* 21, 635-647

104. Lee, C.P. and L. Ernster (1964), *Biochim. Biophy. Acta* 8, 187-190

105. Rydstrom, J., A. Teixeira da Cruz and L. Ernster (1970), *Eur. J. Biochem.* 17, 56-66